ANNUAL REPORT
OF
PROGRAM ACTIVITIES

NATIONAL INSTITUTES OF HEALTH

1958

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

NATIONAL INSTITUTES OF HEALTH
PUBLIC HEALTH SERVICE
U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE

U.S. NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

Report of program activities.

ANNUAL PROJECT REPORT

CALENDAR YEAR 1958

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NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

ANNUAL PROJECT REPORT

Calendar Year 1958

Summary Sheet

LABORATORY OF NUTRITION AND ENDOCRINOLOGY

Estimated Obligations for FY 1959

Total - $1,171,547
Direct - $713,200
Reimbursements - $458,347

Project numbers 1 thru 14 included

Serial No. NIAMD- I
1. Nutrition & Endocrinology
2. Nutrition
3. Bethesda

PHS - NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Metabolic functions of nutrients in experimental animals.

Principal Investigators: Mrs. J. C. Baird, G. N. Briggs, M. [illegible] Spivey Fox, and M. E. Reid

Other Investigators: C. J. Pollard, L. O. Ortis, M. M. [illegible] and Dr. O. Mickelsen

Cooperating Units: Laboratory of Infectious Diseases (Dr. V. T. Haas) NIAID Project No.
Laboratory of Biology (Dr. M. Potter) NCI Project No.
Laboratory of Neurochemistry (Dr. H. [illegible] Agranoff) NINDB Project No.
Ophthalmology Branch (Dr. L. Von Sallmann) NINDB Project No.
Basic Research, (Dr. R. E. Coggeshall) NIMH Project No.

Man Years (calendar year 1958)
Total: 10
Professional: 4 (Includes Mr. M. E. Reid who is a guest worker)
Others: 6

Project Descriptions:

Objectives: To study the biochemistry, physiology, and nutrition of certain essential nutrients (particularly fat-soluble nutrients, lipotropic factors, folic acid, vitamin B12 and certain minerals and amino acids) in experimental animals (chick, guinea pig, rat, and mouse). Specifically, to study the interrelationships of these essential nutrients, to evaluate possible unidentified dietary factors affecting growth of animals, and to determine the specific function in cellular metabolism of vitamins and other essential nutrients whose function is unknown.

Methods Employed: Rats, mice, chicks, and guinea pigs are fed specially prepared highly purified diets composed of all known nutrients (except the material, or material being studied) under carefully controlled conditions. The effect of deficiency is studied by measurement of physiological, chemical, and

enzymological changes in the animal and its tissues. From determinations in the whole animal, attempts are made to relate the changes to altered metabolism at the cellular level. In cooperation with members of other laboratories the relationship of nutrition to other fields of science (neurology, pathology, virology, etc.) is studied.

Major Findings: In the past year some of the significant findings have included:

A. Metabolism of fat-soluble nutrients:

1. Vitamin A. It was reported from England during the past year that vitamin A_1 aldehyde (retinene$_1$) had been isolated from hens' eggs. This was the first evidence that retinene existed in any tissue but the retina, and may be an important lead to the present unknown role of this vitamin in cellular metabolism. Experiments in this laboratory have confirmed this occurrence of retinene$_1$ in certain fish and frog eggs. Furthermore, it was shown that a single fish egg contained retinene$_2$, a pigment not previously found outside of the retina. An esterase from pancreas which effectively esterifies vitamin A alcohol with fatty acids was prepared and the kinetics and specificity determined.

2. Vitamin E. Studies of the metabolic function of vitamin E have shown that chicks can be raised to maturity on a purified diet devoid of vitamin E. Growth and egg-laying were normal even though no vitamin E could be found in the tissues of the birds, nor in the eggs they laid. The activity of DPNH-cytochrome C reductase in the heart particulate fraction from the deficient chicks was identical to that from similar chicks receiving vitamin E. Studies of various respiratory enzyme systems in which vitamin E has been implicated by other workers have failed to show that this vitamin acts in such systems. The reactivation of DPNH-cytochrome C reductase preparations which have been inactivated by extraction with isooctane has been found to be non-specific for vitamin E inasmuch as physical methods (centrifugation or lyophilization) will also reactivate the system. The development of a refined micro procedure for determining vitamin E has shown that d-tocopheryl quinone does not occur in cell fractions or in whole tissues. This finding is further negative evidence for a role of vitamin E in respiratory enzymes.

3. Fatty acids. Studies of unsaturated fatty acid-vitamin E-vitamin A interrelationships have shown that high dietary levels of unsaturated fat markedly reduce body vitamins A and E in the tissues. It has been found that the vitamin E-deficiency syndrome in the rat, encephalomalacia, produced by feeding unsaturated fat, is usually complicated by an accompanying deficiency of vitamin A. Additional studies are in progress to differentiate the central nervous system lesions due to vitamin E deficiency from those due to a lack of vitamin A. The ability of thyroid gland to accelerate vitamin E deficiency (muscular dystrophy) in the chick was found to be due to the combined action of its unsaturated fatty acid content and its mineral

content (ash). The anti-vitamin E effect could be overcomed by an appropriate mixture of unsaturated fatty acid and mineral mixture which stimulates the ash of Torula yeast. In the guinea pig, linoleic acid could completely satisfy the requirement for essential fatty acids.

B. Studies on lipotropic factors:

1. Vitamin B_{12}, choline, and fat interrelationships. Previous studies showed that the vitamin B_{12} requirement of the chick for growth increased as the fat content of the diet increased. Methionine could completely replace the increase in requirement for vitamin B_{12} caused by a high level of dietary fat. Dietary levels of methionine which are tolerated by chicks fed normal diets become highly toxic in fat-free diets. Symptoms were poor growth, perosis (slipped tendon) and inadequate feather formation and pigmentation. Vitamin B_{12} was only partially protective. Fat antihemorrhagic was not grossly affected in the studies since the serum lipids (total lipid, phospholipid, and cholesterol) were unaffected by vitamin B_{12} deficiency or methionine supplementation at 0.1 intakes of 0, .4, or .8% of the diet. Preliminary studies indicate that the effect of a high fat intake upon the vitamin B_{12}-methionine requirement is specific for methionine and does not reflect simply a more efficient utilization of amino acids effected by vitamin B_{12}.

2. Choline and inositol. Chicks fed a purified diet deficient in choline grew poorly and had moderately severe perosis. Supplementation of this diet with 0.2% inositol increased the incidence and severity of the perosis; a 1% level of dietary inositol was still more detrimental. As a result of these experiments and two earlier studies in collaboration with Dr. Agranoff, and from a consideration of past studies relating to growth processes, it is suggested that choline and inositol compete for specific structural phospholipides.

C. Folic acid in relation to disease:

1. Lymphocytic choriomeningitis. Continuation of studies of this virus (in cooperation with Drs. V. H. Rhoades and H. Potter) have shown that the virus kills all strains of mice tested (5) when they are supplied folic acid, but not when the vitamin is omitted from the diet. The addition of ascorbic acid, mixtures of various amino acids, or mixtures of purines and pyrimidines to the folic acid deficient diet did not alter the survival of the infected mice.

2. Leukemia. It is known that folic acid antagonists delay death in mice infected with leukemia. When leukemia cells were injected into folic acid deficient mice, it was found that the time of death was significantly retarded, just as occurs when antagonists are used. However, when a strain of leukemia cells which are not affected by folic acid antagonists was injected into deficient mice, death was delayed significantly.

3. Folic acid deficient diets. Experiments with animals have indicated that it will soon be possible to formulate a diet suitable for man which contains certain components but which is severely deficient in folic acid. This work will be carried on also with studies with leukemia patients.

D. Mineral requirements:

1. Salt mixture for the chick. The information from this laboratory and elsewhere indicated a need to revise the Chick Salts A mixture along the following lines: a) Alter specific mineral levels to meet newly established requirements or to avoid excesses. b) Eliminate or incorporate at minimal levels those salts found to promote oxidative rancidity ($CuSO_4$, $MnSO_4$, and $Fe_3(PO_4)_2$) and the Maillard-type of browning reaction (K_2HPO_4). Of several salt mixtures formulated and tested, one, Salts L, has proven to be very satisfactory. Each required mineral is present at 1.2 to 2 times the best substantiated requirement figures. In a standardized test for the development of oxidative rancidity, the new salt mixture delayed the development of rancidity ten-fold over the time observed with Chick Salts A. Salts L did not promote browning in diets under ordinary conditions. Chicks receiving Salts L in complete casein or soybean protein diets of either low or high fat content have all grown normally. Supplements of molybdenum and selenium, which are not present in Salts L, have not caused any improvement in growth with these diets.

2. Zinc deficiency in the guinea pig. Symptoms of zinc deficiency as indicated by facial sores and hypotheraide were obtained, but a spontaneous recovery occurred later. It appeared that the animals were obtaining enough zinc from some undetermined source even though extreme precautions were taken, such as the use of highly purified diet ingredients, distilled water, and stainless steel cages. This problem will be continued.

E. Studies on amino acid and protein requirements, and unidentified growth factors:

1. Amino acid and protein requirements in the guinea pig. Studies were made with 20, 30, 35, and 40% levels of protein. With casein as the protein, growth increased with each elevation in level up to 30%; with purified soybean protein, graduated increases occurred up to the 40% level. Studies with amino acid supplements furnished an explanation for the apparent high protein requirement. Neither casein nor soybean protein has the optimal balance of amino acids that this animal requires. With casein, arginine is the chief limiting factor and with soybean protein it is methionine. These supplementary amino acids caused marked improvement in growth with the 20% level of protein and even at the 30% level definite increases occurred. With casein in the diet, a marked improvement resulted from the addition of arginine but maximum growth was obtained only after adding a mixture of amino

acids (arginine, methionine, tryptophan); with soybean protein diets, maximum growth could be obtained by either raising the protein level to 40% or by the addition of methionine (1.0% to the 20% protein diet or 0.5% to the 30% diet). In studies of niacin and tryptophan metabolism it was found that with 20 or 30% of casein or 30% of soybean protein the requirement for niacin is 10 mg./kg. of diet. With 20% of soybean protein it is 20 mg./kg. Using amino acid diets, the requirement for L-tryptophan is 1.5 - 2.0 gm./kg. of diet. The D-isomer is used but less efficiently than the L.

Significance to NIAMD Research: Additional basic information on the nutrition, biochemistry, and metabolism of essential amino acids, vitamins, minerals, fatty acids, and unidentified factors in different animals should contribute significantly to our knowledge of the role of these essential nutrients in man. It is becoming increasingly evident that nutrition of man plays a role in the etiology of certain degenerative and metabolic diseases such as arthritis, atherosclerosis, muscular degeneration, chronic abortion, dental defects, diabetes, certain infectious and neurological diseases, and certain types of cancer. Basic studies in nutrition and the biochemistry of nutrients may lead to clues to the prevention or cure of some of these diseases.

Proposed Course of Projects: In the following year special emphasis will be placed on studies designed to explain the effects of nutrients in the whole animal by the metabolic function of these substances at the cellular and enzyme levels.

Part B included: Yes

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Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Bieri, J. G., Briggs, G. M., and Pollard, C. J. Acceleration of vitamin E deficiency by Torula yeast. II. Effect of Torula yeast ash and lipid. Proc. Soc. Exp. Biol. Med. 99: 262-265, 1958.

Briggs, G. M. Nutrition Research. Feedstuffs, December 28, p. 72, 1957

Fox, M. R. Spivey. Nutritional significance of vitamin B_{12}. I. Metabolic functions in experimental animals. World Rev. Nutr. Dietet. (in press).

Fox, M. R. Spivey, and Mickelsen, O. Salt mixtures for purified-type diets. I. Effect of salts in accelerating oxidative rancidity. J. Nutrition (in press).

Pollard, C. J., and Bieri, J. G. The destruction of vitamin A by blood. Brit. J. Nutrition 12: , 1958 (in press).

Pollard, C. J., and Bieri, J. G. Nature of the inactivation by iso-octane extraction of enzymes of the respiratory chain. Biochim. Biophys. Acta 30: 658- , 1958.

Pollard, C. J., and Bieri, J. G. On the occurrence of vitamin A aldehyde in fish and frog ova. Biochim. Biophys. Acta (in press).

Reid, M. E., and Martin, M. G. Nutritional studies with the guinea pig. V. Effects of deficiency of fat or unsaturated fatty acids. J. Nutrition (in press).

Honors and Awards relating to this project:

Borden Award in Poultry Science (gold medal and $1,000) presented to Dr. G. M. Briggs in August 1958 by the Poultry Science Association

Part A.

Project Title: Studies on obese and germ free animals

Principal Investigators: Drs. P. S. Daft, O. Michelsen, Z. Dolgay
R. S. Yamamoto, and Mr [illegible]

Other Investigators: None

Cooperating Units: Section on Fractionation & Isolation (Mr. H. Bakerman) NIAMD Project No. 13
Section on Pathologic Anatomy (Dr. L. Sokoloff) NIAMD Project No 82

Man Years (calendar year 1958)
Total: 9
Professional: 4
Other: 5

Project Description:

Objectives: To determine the biochemical and physiological characteristics of animals made obese by dietary means and the changes resulting from the subsequent loss of weight; to study the nutrition and biochemistry of germ-free animals; to determine the nutritional and biochemical changes that occur in [illegible] exposed to ethylene oxide; to study the nutritional requirements of rabbits.

Methods Employed: Obesity was produced in rats by feeding a high fat diet. The influence of various dietary levels of fat on the production of obesity has been studied. Obese and normal weight rats were starved for periods of 24 to 48 hours during which blood and urine samples were collected for lipid, sugar, and related compounds. The urinary excretion of protein by obese and control rats has been studied when the animals were fed a variety of rations.

The nutritional requirements of germ-free rats and guinea pigs are being studied. A variety of foods for maintaining germ-free animals and a number of methods of sterilizing food and equipment have been studied.

The reaction of ethylene oxide with a number of nutrients has been studied. This work has been extended to blood plasma.

Major Findings: (1) Obesity. Obesity in some strains of rats can be produced by feeding the animals a high fat diet on an ad libitum basis. The rate of growth and the consequent development of obesity are proportional to the level of fat in the diet over the range 10 to 40% (above the latter level growth is not accelerated). A hybrid strain of rat recently developed at NIH (SBS/S) is resistant to this type of obesity.

There appears to be a slight disturbance in carbohydrate metabolism in the obese rats as evidenced by the delay in the lowering of the blood sugar following an intravenous glucose tolerance. This abnormality was also present in the rats which had been made obese by Ingle's technique (giving water with a high carbohydrate diet so that the animal has to eat when it is thirsty). In all the obese rats the rate of removal of the glucose was inversely related to body weight. The obese rats had higher plasma lipid and phospholipid levels than their littermate controls. During a 36-hour starvation period the plasma lipid levels in the obese rats decreased slightly whereas those in the controls increased.

Normal male rats fed a commercial stock diet routinely show considerable amounts of protein in the urine whereas females have very little. The amount of protein in the urine of male rats increases with age and protein intake, whereas the excretion in female rats normally does not change. Obese rats one year of age or older excrete more protein than their normal weight controls with the greatest percentage increase occurring in the females. Although the above factors (age and protein intake) increase protein excretion, no dietary procedures have been known to reduce the protein excretion. Recently it was observed that when the caloric density of the high fat diet was reduced by adding an inert filler (cellophane) the male rats fed the diet did not show the increase in urinary protein with age characteristic of animals fed the stock diet.

(2) Germ-free animals. When germ-free rats were fed a diet containing 22% casein but no folic acid, they developed typical folic acid deficiency symptoms. A similar deficiency can be produced in conventional rats only when the protein level in the diet is reduced and sulfonamides are added. The germ-free rat deficient in folic acid shows no improvement when fed on amino acid mixture (threonine, methionine, and tryptophan) which cured conventional rats.

Germ-free guinea pigs fed a sterilized stock diet have enlarged ceca which, with their contents, may amount to 30% of their body weight. Conventional guinea pigs fed the same sterilized diet have ceca which are only 5% of body weight. When germ-free guinea pigs were fed a sterilized diet prepared from purified ingredients, the ceca represented 5% of the body weight. Adding autoclaved cereals to the purified diet increased the size of the ceca. The amount of [illegible]

(3) Ethylene oxide. The reaction product of ethylene oxide and nicotinamide was isolated and characterized as N'-1-(2-hydroxyethyl) nicotinamide. When an alkaline solution of this compound is irradiated with ultra-violet light, it fluoresces to an extent that is about twice that of N'-methylnicotinamide.

The Committee on Food of the National Research Council requested cubic mole sample of lyophilized plasma that had been treated with ethylene oxide as a means of destroying the [illegible] virus. The treated plasma showed a destruction of 90% lysine, 70% histidine, 30% tryptophan, and 20% phenylalanine with practically no destruction of arginine. These findings were totally unexpected since the moisture content of the plasma was reported to be less than 1.0% and at these low levels of moisture the ethylene oxide reactions occur to only a slight extent in other products.

(4) Alkali treatment of corn. Work in this and other laboratories showed that some of the niacin in corn was available to rats only after treatment with dilute alkali. A similar situation exists for the rat when the corn in the usual pellagragenic ration was reduced from 72% to 18%. On this ration the rats receiving the alkali-treated corn gained 57 grams in 35 days while those on the untreated corn gained 22 grams.

(5) Nutritional requirements of rabbits. Rabbits gained 25 grams per day on a 20% protein diet when soybean meal was the source of protein. On this same diet with 20% casein as the protein source the rabbits gained 16 grams per day. The explanation for the poor growth on the soybean meal does not appear to be differences between the diets in amino acids or inorganic constituents. Tests with another purified diet suggests that the kind of carbohydrate and salt mixture appears to be involved in the above.

Significance to Military Medicine: [illegible] is a major health problem in the United States. The obesity developed in the rats here closely resembles that in human beings since it is produced entirely by dietary means.

The germ-free studies are beginning to show the importance of the intestinal flora to health and well-being and the interrelation between diet and the changes that occur in the gastrointestinal contents in the absence of bacteria.

Proposed course of the project: Work on the [illegible] will be continued in an effort to determine the changes in the composition of organs and tissues as the animals become obese. Further studies will be made on the factors that appear to influence proteinuria in the rat. The work with germ-free animals will probably be expanded during the coming year to other areas than the nutritional studies.

Form C.R. [illegible]

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Part D: Honors, Awards, and Publications

Publications other than abstracts from this project:

Michelsen, O. Age changes in body composition. Public Health Reports 73: 295-301, 1958.

Michelsen, O., and Anderson, A. A. A method for preparing intact animals for carcass analyses. J. Lab. Clin. Med. (in press).

Contacy, G. R., Michelsen, O., Sargeus, D. W., Young, M. D., and Pirkle, C. I. Chloroquine or pyrimethamine in salt as a suppressive against sporozoite-induced vivax malaria (Contacy strain). Bull. World Health Org. 19: 53-67, 1958

Honors and Awards relating to this project:

None

PHC - NIH
Individual Project Report
Calendar Year 1958

Part I

Project Title: Diabetes - Effect of hormones on metabolism of carbohydrates, fat and protein.

Principal Investigators: Drs. R. O. Scow and S. S. Chernick

Other Investigators:

Cooperating Units:

Man Years (calendar year 1958):
Total: 6
Professional: 2
Other: 4

Project Description

Objectives: To study the influence of hormones on diabetes and on the metabolism of carbohydrate, fat and protein; to study factors, in addition to hormones, that regulate fat mobilization, fat oxidation and ketone body formation; [illegible] the role of the anterior pituitary and adrenal glands [illegible] various phenomena which occur in experimental diabetes.

Methods Employed: Experimental animals deprived of one or more endocrine glands are treated with [illegible] hormones. The effect of the extirpations and of treatments [illegible] are studied in vivo and in vitro experiments, using conventional and isotopic techniques.

Major Findings: Although the classical experiments [illegible] (1931) and Long and Lukens [illegible] that the anterior pituitary and adrenal glands are [illegible] the development of diabetes in pancreatectomized animals, [illegible] few studies [illegible] which hormones of these glands are [illegible] responsible [illegible] development of ketosis and acetonemia in the [illegible] rat.

The key role [illegible] and adrenal hormones in diabetes [illegible] 99.5% of its pancreas. [illegible] of the rats were also hypophysectomized or adrenalectomized [illegible] was found that these rats were [illegible]

insulin, developed ketosis only if given cortisone or if the secretion of the adrenal cortex was stimulated by ACTH injections. Hyperglycemia and glycosuria occurred only in rats that developed ketosis. Growth hormone, up to 30 mg./kg., had no effect on blood glucose, ketone bodies or lipids in the doubly operated rats. ACTH produced ketosis only when the adrenal glands were intact. The ketogenic action of cortisone and ACTH was not seen in rats with an intact pancreas.

The final level of blood ketone bodies, ranging up to 250 mg.%, was closely related to the amount of triglycerides accumulated in the liver during the 2-day experiment. The glucocorticoid not only increased ketosis but also raised the level of fat in the liver. It is suggested that glucocorticoids increased the amount of ketone bodies formed in the diabetic by increasing the release of fat from adipose tissue with a consequent accumulation of hepatic fat and an acceleration in the rate of ketogenesis. These effects of the glucocorticoids are prevented by insulin.

Hypophysectomized-pancreatectomized rats deprived of insulin died in diabetic ketosis within 24 hours when given 4 mg. of cortisone. Similar effects were obtained when 20 gamma of delta-1, 9 alpha fluoro, 16 alpha methyl hydrocortisone (dexamethasone) were injected. The terminal blood ketone body levels in these rats were as high as 250 mg.%. The minimal amount of the latter hormone needed for the development of ketosis in the "Houssay" rat was 2 gamma; that of cortisone was 500 gamma. In fed pancreatectomized rats given inadequate amounts of insulin, the diurnal variation in blood triglycerides and blood ketone bodies was increased about 10-fold when dexamethasone was also injected. These adverse effects of dexamethasone are prevented by insulin.

In the above studies, dexamethasone produced the same effects in the "Houssay" rat as did cortisone acetate: ketosis, accumulation of triglycerides in blood, liver and kidneys, hyperglycemia and glycosuria. In these respects, dexamethasone appears to be about 200 times as potent as cortisone. It is of interest that recent studies have indicated that dexamethasone is about 60 times as potent as cortisone as an antirheumatic agent.

Preliminary observations demonstrated that pregnant rats develop a marked degree of ketosis and hyperlipemia if fasted during the last 3 days of pregnancy. The blood ketone bodies are increased to 80 mg.% and the triglycerides to 500 mg.%; the phospholipids and cholesterol levels are little changed. An extensive study has been made of some of the factors, especially hormonal, involved in this process. The severity of the condition increases with number of fetuses. It has been found that administration of glucose immediately corrects the ketosis and hyperlipemia. Extirpation of the uterus with the placentae and fetuses immediately lowers the blood lipids, mainly triglycerides, but has little effect on the ketone body level.

accumulation of fat in the liver and [illegible] only decreases mobilization of fat. [illegible] hormones, well-known for their ability to mobilize fat, [illegible] bovine growth hormone, cortisone, and dexamethasone as well as injections of large [illegible] effect in preventing [illegible] fast.

The interrelationship of growth hormone and thyroxine in the growth of muscle, [illegible] and chemical analyses, in young rats deprived of both the thyroid and pituitary glands. It has [illegible] stimulated growth of all tissues, thyroxine stimulated growth of only muscle, skeletal tissues and skin. In some tissues the growth response to thyroxine had just as pronounced as that to growth hormone alone. One of the specific effects of growth hormone was in the stimulation of the incorporation of collagen formation in muscle, bone and skin. In certain tissues administration of both hormones produced greater growth than was either hormone; however, the weight gain in doubly treated rats equalled the algebraic sum of the gains in the singly treated groups.

Significance to Diabetes Research: The chief problem facing the diabetic patient at the present [illegible] pathological processes involved in the complications and degeneration of fat metabolism, particularly atherosclerosis and Kimmelstiel-Wilson disease. The nature of the disturbances of fat metabolism in diabetes is poorly understood. The present observations not only demonstrate the utility of the rat [illegible] the important role of the adrenal cortex, in particular the "glucocorticoid" secretion, in the regulation of fat in the body. The marked accentuation of the diurnal variations of blood lipids in the poorly treated pancreatectomized rat when given glucocorticoids suggest a new experimental approach for the study of the relation between fat metabolism and vascular degeneration.

Proposed Course of the Project: The [illegible] studies of fat metabolism in [illegible] and normal rats will be continued. [illegible] section of glucocorticoids on fat [illegible] the insulin sensitive [illegible] other factors on ketosis in fasting pregnant rats is still under study. An investigation [illegible] of the effect of growth hormone and thyroxine on skeletal muscle of thyroidectomized-hypophysectomized rats to see if the increased myosin content in treated animals is accompanied by increased work performance by the muscle.

Part B included Yes [X] No [ ]

Serial No. [illegible]

[illegible]- NIH
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Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Scow, R. O., Chernick, S. S., and Smith, B. B. Ketosis in the rat fetus. Proc. Soc. Exp. Biol. Med. 98: 833-835, 1958.

2. Chernick, S. S., and Scow, R. O.: Early effects of "total" pancreatectomy on fat metabolism in the rat. Am. J. Physiol. 196: , 1959.

3. Scow, R. O., Chernick, S. S., and Guarco, D. A.: Ketogenic action of pituitary and adrenal hormones in pancreatectomized rats. Diabetes 8: , 1959.

4. Scow, R. O.: Effect of growth hormone and thyroxine on growth and chemical composition of muscle, bone, and other tissues in thyroidectomized-hypophysectomized rats. Am. J. Physiol. (in press).

Honors and Awards relating to this project:

None

Experimental
3. Bethesda

Part A:

Project Title: Biochemistry of Factor 3 and Other Selenium Compounds

Principal Investigator: Klaus Schwarz

Other Investigators: Calvin M. Foltz

Cooperating Units: Analytical Service Laboratory, LC, NIAMD (?)
National Nutrition Research Institute, Pretoria, South Africa

Man Years (calendar year 1958).
Total: 4-1/3
Professional: 2
Other: 2-1/3

Project Description:

Objectives: To study the biological effects of Factor 3 and other selenium compounds, to investigate their natural distribution, determine their chemical nature and their biological specificity. To differentiate and delineate Factor 3 deficiency diseases from other deficiencies, especially vitamin E deficiency. To isolate and characterize Factor 3 and to devise methods for the chemical synthesis of Factor 3-active compounds.

Methods Employed: Biopotency of selenium compounds and Factor 3 is determined by animal assays, using protection against dietary liver necrosis in the rat as test system. A microanalytical method, radio activation analysis, and a highly sensitive colorimetric assay are used for selenium determinations. Synthetic methods are applied for the preparation of various selenium compounds, especially of selenium containing sulfur amino acid derivatives. Fractionation and isolation techniques (chromatography, countercurrent distribution, etc.) are used for the further fractionation of Factor 3 preparations from natural sources, and also for the purification of other natural compounds.

Major Findings: Comparison of the biopotencies of numerous selenium containing organic substances has shown that the great majority affords protection at selenium levels which are equivalent to those of selenite selenium (50 per cent effective dose, ED_{50}= 2-3 µg Se in the diet). The observation that Factor 3 is 3-5 times

Part B included Yes [X] No. [ ]

as potent was strengthened by further, comprehensive tests. A few synthetic organic selenium compounds have been found to be more effective than selenite, but none of them reaches the biopotency of Factor 3. Comparison of a series of synthetic diselano di-acids of the general formula $HOOC-(CH_2)_n-Se-)_2$ showed that there is a distinct maximum for biopotency at the 5-carbon chain length. However, "activated" selenium methionine (5-adenosylselenomethionine), studied in cooperation with Dr. Shah of NIAMD, was less potent against dietary liver necrosis than selenite. 6-Selenoctic acid, the selenium analogue of thioctic acid, was also found to be of lesser biopotency.

A substance containing selenium bound to sulfur, bis-(2-nitro-5-methyl-phenylselenenyl) sulfide, was more potent than selenite. Sulfur amino acids, especially L-cystine, produced from natural sources, were found to be contaminated with small, but biologically important amounts of selenium. Methods were developed to eliminate the element. The biopotency of L-cystine, the first compound ever identified to protect against liver necrosis, is partially due to the trace contamination with selenium; up to 2 µg of the element were found per g of commercial L-cystine.

The structure of the reaction product of selenium dioxide and cysteine has been a matter of controversy. Both the di-cysteine form, $Se(S-CH_2-CHNH_2-COOH)_2$, derived from the hypothetical selenium mono-oxide, and the tetra-cysteine form, $Se(S-CH_2-CHNH_2-COOH)_4$, derived from SeO_2 were postulated. New methods for the preparation of these two categories have shown conclusively that both types of compounds exist. The selenium di- and tetra- derivatives of cysteine, homocysteine, glutathione, lipoic acid, etc. were prepared. None of these was as potent as Factor 3 in the bioassay; this study may relate to the binding of SeO_2 to SH groups of proteins.

A report from elsewhere implied that arsenic, cobalt and molybdenum possess a sodecreasing effect on the development of the disease. In our experimental system no such influence was detectable.

The older fractionation procedure for the preparation of concentrated Factor 3 from yeast kidney powder contained steps which produced profound changes in the Factor 3 molecule. The main component of Factor 3 fraction free with selenium has the properties of an organic selenium compound and is unstable. During the current year a new, less rigorous method for the concentration of Factor 3 has been devised which should lead to the isolation of the originally present biologically active form.

Selenium poisoning, responsible for widespread losses of cattle and sheep in fifteen states, is produced by selenium accumulator plants, mainly belonging to the vetch family (Astragalus). The toxic principle of Astragalus has been investigated. Selenium is

present in low molecular, but organically bound form. It was readily extractable with H_2O and alcohol. The concentrated Astragalus principle was tested in the biocassay against liver necrosis. It showed a biopotency only slightly inferior to that of selenite. Tschesche postulated recently that the structure of the compound is that of Se-methylselenocysteine. According to our results the compound may be a selenonium salt or the selenamide derivative of Se-methylselenocystine.

Dietary experiments with vitamin E deficient, but Factor 3 supplemented, Torula yeast diets in rats have led to a new type of vitamin E deficiency disease, characterized by profound lesions of the central nervous system. Ataxia and a palsy-like syndrome develops accompanied by a serious paralysis and cramps of the extremities. The lesion occurs with 100% incidence after approximately 6-9 months. Histologically, widespread disturbance of the myelinization of the white matter of the brain, the spinal cord and also of peripheral nerves is found. The lesion is currently under study in collaboration with Dr. Angel Tschesche of the Armed Forces Institute of Pathology.

Supplementation of the basal diet with vitamin E, on the other hand, leads to a clear cut Factor 3 deficiency. The more important signs of this disease are lack of growth, muscular wasting, adrenal atrophy and widespread pancreatic dystrophy. The disease easily is preventable by the addition of 4-6 ug% of selenite-selenium to diet.

The differentiation of various deficiency diseases by effects of Factor 3, on one hand, as compared to effects of vitamin E, on the other hand, has shown that there are certain diseases which depend entirely on tocopherol, while others are primarily caused by lack of Factor 3-selenium. Vitamin E dependent are resorption sterility in the rat, and encephalomalacia in the chick. Muscular dystrophy in the rabbit was also found to belong into this group. When produced in young rabbits on a vitamin E-free soy bean diet, Factor 3-active selenium compounds did not influence the course of muscular dystrophy.

The effect of sulfur amino acids on dietary liver necrosis has been reevaluated. Selenium-free sulfur amino acids have been found to delay the development of liver necrosis for approximately 10 days without, however, protecting the animals from the fatal outcome. When methionine, homocystine, cystine and cystine were added in suboptimal levels of Factor 3, selenite or tocopherol, they suppressed the requirement for liver protecting substances. Animals were completely protected with levels of Factor 3, selenite, or vitamin E which otherwise were ineffective. To elicit the protecting effect .8-.6% of the sulfur amino acids are required. Methionine sulfoxide gave only a partial effect, and the sulfone was ineffective. Serine and

homoserine were without influence. The moderating effect may be related to cystamine, which has been found to spare tocopherol or Factor 3 more efficiently than the S-amino acids. Liver coenzyme A levels were unaffected by cystamine supplements to the diet.

A double blind study on the effect of Factor 3-active selenium in kwashiorkor has been carried out in collaboration with the National Nutrition Research Institute in Pretoria, South Africa. A total of six cases was treated with seleno-cystathionine, mixed into lactose, another six cases received lactose only. There was a distinct advantage of the Factor 3 supplemented group.

Significance to NIAMD Research: The studies have yielded results of basic significance for the understanding of biochemical and nutritional phenomena. Factor 3, a potent biochemical agent, is effective in several species in preventing fatal necrotic lesions of liver, heart, kidney, muscle and other tissues. The discovery that Factor 3 is a selenium compound has opened up numerous questions of scientific interest and has also practical implications. It may lead to a significant contribution to the understanding and the treatment of human diseases in the human.

Proposed Course of Project: Further experiments to clarify the relation between the chemical structure of selenium compounds and their biopotency against liver necrosis are under way. The current efforts at fractionation of Factor 3, directed toward the isolation of the biologically active, unaltered form of Factor 3 should be brought to conclusion. This will be followed by the determination of the structure of Factor 3 and ultimately by attempts at the synthesis of Factor 3. The studies will be supplemented by synthesis of other selenium compounds of a particular interest with regard to Factor 3 potency. The experiments will be coordinated with enzymatic investigations on the mode of action of Factor 3-selenium. The incorporation of radioactive selenium in tissue will be studied. The binding sites for selenium will be determined. The reaction mechanisms between inorganic selenium compounds and proteins shall be investigated. Factor 3-selenium deficiency in animals will be further investigated with the aid of diets other than the Torula yeast ration hitherto used. The question whether the relationship between Factor 3 and Factor 3-selenium potentiated the dose response against liver necrosis is being examined.

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Schwarz, K., Dietary Necrotic Liver Degeneration: An Approach to the Concept of the Biochemical Lesion. "Liver Nutrition", Ralph W. Kruger, Ed., Am. Inst. of Biol. Sci., Washington, D. C., p. 509, (1958).

2. Schwarz, K., and Foltz, C. M., Factor 3 Activity of Selenium Compounds. J. Biol. Chem. 233, 245, (1958).

3. DeWitt, W. B., and Schwarz, K., Multiple Dietary Necrotic Degeneration in the Mouse. Experientia 14, 88, (1958).

4. Burris, R. L., Ludwig, M. I., and Schwarz, K., Ineffectiveness of Factor 3-Active Selenium Compounds in Absorption-Excretion Bioassay for Vitamin E. Proc. Soc. Exptl. Biol. and Med. 97, 686, (1958).

5. Howe, E. E., Fry, G. E., and Schwarz, K., Ineffectiveness of Factor 3-Active Selenium Compounds in Muscular Dystrophy of Rabbits on Vitamin E-free diets. Proc. Soc. Exptl. Biol. and Med. 98, 27, (1958).

6. Schwarz, K., Effect of Antioxidants on Dietary Necrotic Liver Degeneration. Proc. Soc. Exptl. Biol. and Med. 99, 27, (1958).

7. Harts, W., and Schwarz, K., The Reversal of Respiratory Decline in Necrotic Liver Degeneration by Nitrogenital Antioxidants. Proc. Soc. Exptl. Biol. and Med. 98, 808, (1958).

8. Schwarz, K., Foltz, C. M., and Bergman, C., Factor 3 and 6-Selenouric Acid. Acta Chem. Scand. 12, 1330, (1958).

9. Schwarz, K., Delaney, J. F., and Foltz, C. M., Relation Between Selenium Tumors in L-Cystine and Protection Against Dietary Liver Necrosis. Metabolism (in press).

10. Schwarz, K., Pajunski, H. R., and Foltz, C. M., Ineffectiveness of Molybdenum, Osmium and Cobalt in Dietary Necrotic Liver Degeneration. Nature (in press).

11. Schwarz, K., Der Faktor 3, das Selen, und die Lebensnotwendigkeit Mehrwertiger Elektrolyte. Vitalstoffe, Mineralstoffe, Spurenelemente (in press).

12. Schwarz, K., Factor 3, Selenium and Vitamin E. Mitchell, Nutrition Reviews (in press).

Honors and Awards relating to this project:

Individual Project Report
Calendar Year 1958

Serial No. NIAMD- 5
1. Nutrition and Endocrinology
2. Experimental Liver Diseases
3. Bethesda

Part A.

Project Title: Investigations on the Dietary Glucose Tolerance Factor (GTF) and a New Agent from Liver (LA) Enhancing Glucose Uptake.

Principal Investigator: Walter Mertz

Other Investigators: Klaus Schwarz

Cooperating Units: None

Man Years (calendar year 1958):
Total: 3
Professional: 1-1/3
Other: 1-2/3

Project Description:

Objectives: To purify and identify the glucose tolerance factor (GTF), to investigate the effects of GTF deficiency in experimental animals, to study the mechanism by which GTF enhances glucose tolerance in intact rats and to assay its effects on glucose uptake in various in vitro systems.

To study the role of liver in glucose utilization and to elucidate the mode of action of a newly discovered humoral agent from liver (LA) which enhances peripheral glucose uptake.

Methods Employed: Male Sprague-Dawley rats are maintained on various natural and semi-synthetic diets, and glucose removal rates are measured after intravenous glucose injection. GTF potency is determined by comparison of glucose tolerance before and after application of a single dose of GTF by stomach tubing. Chemical and physical fractionation procedures (adsorption, elution extraction, chromatography and countercurrent distribution) are used to purify the factor.

Rat liver slices are depleted of glycogen by incubation in carbohydrate free media. Glucose utilization and other metabolic functions of such slices are studied, with and without addition of GTF.

Part B included Yes [X] No [ ]

Protein-free water extracts are prepared from livers and other organs, and their effect on glucose uptake is determined in vivo and in various in vitro systems.

He-La cells in tissue culture are incubated in a glucose containing medium, with and without addition of purified GTF preparations and other supplements.

Major Findings: Normal glucose removal rates of 4-4.5%/min. were found in rats on two natural diets, namely kitchen scraps, and the McCollum wheat-casein ration. Torula yeast diets, on the other hand, did not maintain normal glucose tolerance. It was detected that three commercial stock rations, namely Purina Laboratory Chow, Hunt Club Dog Food and Rockland Rat Diet are seriously deficient in GTF. In animals on these diets glucose removal rates declined to around 2.5%/min. This decline was prevented by dietary supplementation of known sources of GTF or by purified GTF preparations. The fully developed defect could also be cured within 18 hours by application of GTF concentrates via stomach tube. This is used as assay for GTF concentrates derived from pork kidney powder and other source materials by fractionation.

Previous findings had indicated that GTF affected glucose removal from the blood only in the presence of the liver. The influence of GTF concentrates on glucose utilization in liver slices was studied. GTF did not affect glucose concentrations in the Warburg medium after two hours of incubation when fresh liver slices were used. Predepletion in a glucose-free medium, however, produced slices which displayed net uptake of glucose from the medium of approximately 50 µg/100 g of tissue and hour. Addition of GTF preparations of known activity to the system increased glucose uptake under these conditions. The specificity of the effect remains to be established. The system may respond to a variety of influences.

In previous investigations it had been discovered that the liver contains an agent which has a profound influence on intravenous glucose tolerance in animals. In GTF deficient animals, small amounts of a potent extract from rat liver will reconstitute normal glucose removal when injected simultaneously with the glucose during the tolerance test. The liver agent is effective in the hemidiaphragm. From 5-20 µg of a crude preparation will increase glucose uptake by ca 40%. In contradistinction to the dietary GTF the liver principle furthermore effects glucose removal in hepatectomized animals.

In the glucose-depleted liver slice described above, crude preparations of LA were found to be effective. 20 µg of an acetone extract produced on the average a three-fold increase of glucose uptake.

Removal of glucose from tissue culture media was investigated with HeLa cells. There was no effect of purified GTF preparations, but the liver agent increased glucose disappearance from the culture fluid significantly by approximately 20%.

Significance to NIAMD Research: The impairment of intravenous glucose tolerance, so far the only known symptom of GTF deficiency, can be considered as a sensitive indicator of an altered metabolic situation of the organism. Although the site of the disturbance in metabolism has not yet been located and no other severe deficiency symptoms have been detected, the GTF may be an essential dietary ingredient. Since impaired intravenous glucose tolerance is one of the earliest signs of diabetes in the human, prolonged GTF deficiency may contribute to the development of a diabetic situation. The finding that stock diets (including the Purina formula used at the Animal Production Section) are deficient in GTF may lead to new standards and a readjustment of the formulation, with an improvement of animal production and quality. The studies with the liver agent offer a new interpretation of the role of the liver in the regulation of blood sugar levels. This may have significance not only under normal conditions but also in various diseases.

Proposed Course of the Project: The attempts to purify and isolate GTF are being continued with the aim to clarify its chemical nature and to make it available for clinical research. For the purification, suitable *in vitro* test procedures are necessary. Therefore, emphasis will be placed on the development of *in vitro* methods for GTF testing. The influence of GTF on fat metabolism is under study. Dietary experiments are in progress to detect whether prolonged GTF deficiency causes other more serious pathological damage. Studies of the mode of action of GTF with isotopically labelled glucose are anticipated.

An investigation into the mode of action of the liver agent, especially a comparison of the latter to insulin, will be carried out concurrently with the studies on GTF. Preliminary to chemical fractionation, attempts will be made to find source materials more abundant than rat liver, and to establish the main chemical and physical properties of the active ingredient, which is apparently of low molecular weight. A comparison of the various *in vitro* systems will be made to determine which is most suitable for LA testing.

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Mertz, W., and Schwarz, K., Relation of Glucose Tolerance Factor to Impaired Intravenous Glucose Tolerance of Rats on Stock Diets. Am. J. of Physiol. (in press).

2. Schwarz, K., and Mertz, W., The Glucose Tolerance Factor and its Differentiation from Factor 3. Archives of Biochem. and Biophys., 72, 515, (1957).

3. Schwarz, K., and Mertz, W., A Note on the Glucose Tolerance Factor (GTF), a New Dietary Agent. "Liver Function", Ralph W. Bremer, Ed., Am. Inst. of Biol. Sci., Washington, D. C., p. 535, (1958).

4. Schwarz, K., and Mertz, W., The Terminal Phase of Dietary Necrotic Liver Degeneration in the Rat. Metabolism (in press).

Honors and Awards relating to this project:

None

PHS-NIH
Individual Project Report
Calendar Year 1958

Serial No. NIAMD- 6
1. Nutrition and Endocrinology
2. Experimental Liver Diseases
3. Bethesda

Part A:

Project Title: The Role of Vitamin E and Factor 3 in Metabolism, and their Relation to Dietary Necrotic Liver Degeneration.

Principal Investigator: Laurence M. Corwin

Other Investigators: Marie N. Lipsett (Guest) and Klaus Schwarz.

Cooperating Units: None

Man Years (calendar year 1958):
Total: 2-2/3
Professional: 2
Other: 2/3

Project Description:

Objectives: To elucidate the mode of action of Factor 3, and also of vitamin E, in intermediary metabolism. To clarify the chain of events in the development of dietary necrosis.

Methods Employed: Mitochondria and microsomes were prepared by differential centrifugation according to the method of Schneider and Hogeboom. Succinic acid oxidation and oxalacetic acid decarboxylation were studied in the Warburg respirometer. DPNH and succinate-cytochrome c reductase activities were measured spectrophotometrically. Oxalacetic acid and pyruvate were measured as their 2,4-dinitrophenylhydrazones.

Major Findings: The discovery of a DPN-dependent decline in succinate oxidation in liver mitochondria of rats fed a vitamin E-deficient diet was reported. Since then experiments have been performed to ascertain the role of vitamin E in this system. Intraportal injection of α-tocopherol into deficient rats will prevent the decline within 30 minutes of injection. The dose of tocopherol needed for this effect was higher than that required for prevention of respiratory decline in liver slices. There was no difference in DPNH-cytochrome c reductase activity in rats on vitamin E-supplemented or deficient diets. This raises the question whether Nason's finding concerning the reactivation of DPNH-cytochrome c reductase by tocopherol in isooctane-extracted or aged muscle preparations is of practical significance.

Part B included: Yes (X) No ( )

Accumulation of oxalacetate was strongly implicated as the cause of the decline of succinate oxidation in the vitamin E-deficient mitochondria. The necessity for NPN for the difference in decline to occur is explained by its known effect in accelerating oxalacetate formation. Compounds known to accelerate the removal of oxalacetate alleviate the inhibition: acetyl CoA, manganese and magnesium ions, and ATP were all effective.

It was observed that ITP did not alleviate the decline of succinate oxidation, although ITP is known to activate oxalacetic acid decarboxylases which act at pH 7.5. The lack of effect of nucleotides to stimulate the oxalacetate decarboxylation in rat liver mitochondria led to the discovery of a new oxalacetic acid decarboxylase which is independent of nucleotides and has an optimum pH of 7.5. This accounts for the lack of effect of ITP on the decline of succinate oxidation.

Arsenite in amounts sufficient to inhibit pyruvate oxidation was without influence on the rate of decline in deficient or supplemented mitochondria. This eliminates the possibility that vitamin E is acting on pyruvate oxidation or citrate synthesis to prevent the decline in succinate oxidation. It was observed that generally the vitamin E-deficient mitochondria in general had higher oxalacetic acid decarboxylase activity than the supplemented mitochondria. Since the succinate-cytochrome c reductase activity also is elevated and parallels exactly that of the decarboxylase, the deficient mitochondria may be damaged and thus more permeable to oxalacetic and succinic acids.

Although there are certain differences, the decline in succinate oxidation in the presence of NPN parallels the respiratory decline of liver slices. Factor 3 has shown to be partially effective in preventing respiratory decline of rat liver slices although it is without effect on the succinate system. Thus since an effect on some oxidative pathway is indicated, a reevaluation of the various pathways has been initiated by comparing liver fractions of rats deficient in Factor 3 to those from supplemented animals. Aerobic glycolysis has been studied by measuring the formation of total pyruvate and lactate from glucose. No significant differences were found in analogy to previous studies with vitamin E.

A separate investigation dealt with the isolation and identification of lipid soluble phenolic compounds from natural sources. These compounds, especially hydroquinones, are biologically important, most likely in electron transfer. They also have "antioxidant" properties. The metabolically active conversion products of vitamin K or vitamin E, for instance, may belong into this category. Thus far, a lipid extract from yeast has been used as a starting material. From this a group separation of phenolic compounds was carried out by extra-

bicarbonate-sodium hydroxide fractionation. After solvent fractionation and countercurrent distribution, several substances have been isolated in milligram quantities, some of them with interesting physicochemical properties. The biological significance of these materials, and their potency as antioxidants is to be established.

Significance to NIAMD Research: Elucidation of the mode of action of vitamin E and of Factor 3 would be of significance. It would be of basic importance for the understanding of normal oxidative pathways, the main source of energy for the life of animals. Clarification of the primary metabolic lesion and the chain of events leading from dietary deficiency of Factor 3 and vitamin E to acute necrosis and death, may produce a more general understanding of necrosis, i.e., death of tissue, and may lead to new approaches in the therapy of such lesions.

Proposed Course of Project: Current investigations will be extended to include a study to determine the reversability of the reaction catalyzed by oxaloacetic decarboxylase, as well as its relation to vitamin E function. The biochemical basis for the damage to all particulates attending vitamin E and Factor 3 deficiency will be investigated. With tissues from animals with or without Factor 3, screening of larger areas of oxidative pathways in intermediary metabolism, for instance, fatty acid oxidation and hexosemono phosphate shunt, is in progress. A study of the effects of selenite on growth and metabolism of yeast is scheduled and also experiments with radioactive selenium, to investigate the conversion of the latter to Factor 3, which may aid in its chemical identification.

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project

1. Corwin, L. M., and Schwarz, K., An Effect of Vitamin E on the Regulation of Succinate Oxidation in Rat Liver Mitochondria. J. Biol. Chem.,(in press)..

2. Schwarz, K., Mertz, W., and Simon, E. J., In Vitro Effects of Tocopherol Metabolites on Respiratory Decline in Dietary Necrotic Liver Degeneration. Biochim. et Biophys. Acta. (in press).

3. Piccardo, M. G., and Schwarz, K., The Electron Microscopy of Dietary Necrotic Liver Degeneration. "Liver Function" Ralph W. Bramer, Ed., Am. Inst. of Biol. Sci., Washington, D. C., pp. 528, (1958).

4. Piccardo, M. G., The Spatial Relations of the Endoplasmic Reticulum to Mitochondria in Liver Cells. "Liver Function" Ralph W. Bramer, Ed., Am. Inst. of Biol. Sci., Washington, D. C., p. 198, (1958).

Honors and Awards relating to this project:

None

Calendar Year 1956

Serial No. [illegible] 7
1. Nutrition and Endocrinology
2. Endocrinology
3. Rodents

Part I.

Project Title: [illegible] - Electrophysiology of the brain, and studies influencing the central physiology.

Principal Investigators: Dr. Anderson, Dr. Wilson, Dr. Fabre and Dr. Knowlton

Other Investigators: Dr. [illegible] (Pharmacology), Dr. Burke (NIMH), Dr. [illegible] (NIH), Mr. Eddy, Dr. Ison and Mr. Hagen

Cooperating Units: Georgetown University School of Medicine, Walter Reed Army Institute of Research, and Armed Forces Institute of Pathology.

Man Hours (Calendar Year 1956)
Total: 4
Professional: 2
Other: 2

Project Description:

Objective: To obtain certain evidence for the hypothesis which is given in part below, namely: that there is a neurohormonal hypothalamo-pituitary activating system closely linked to the vegetative integrating system of Hagoort.

Methods employed: Determination of areas in the midbrain reticular fog by a neurosurgical approach and by electrolytic lesions as well as ablation of the area by stereotactic techniques. ACTH release is measured by quantitative assay of urinary corticoids. Methods for the determination of corticoids in dog and rat urine have been developed in this laboratory. ACTH release is also measured by radioactive depletion rate from the thyroid. Cortisol rate in the urine was determined by a modification of the von Euler method.

Major Findings: [illegible] lesions in the midbrain [illegible] over the normal in urinary volume [illegible] while in other cases no change [illegible] occurred. The emotional state [illegible] striking changes in EEG pattern, in some of [illegible] observed.

In a series of dogs [illegible] upper midbrain [illegible] In a number of dogs in which [illegible] opposite effect was noted, [illegible] in response to stress. [illegible] changes of the urinary [illegible]

Significance to the program [illegible] influences on metabolism [illegible] in the study of human metabolic [illegible]

Proposed course of [illegible] the [illegible] the [illegible] squirrel monkeys are being studied.

Part B included.

Park E.

Publications other than abstracts from this project:

Pahm, E., Purtz, J., Andersen, E., and Soloman, E.
Morphologic and functional changes associated with transplantable ACTH-producing pituitary tumors of mice. Am. J. Path. 33, 1073-97, 1957.

Pahm, E., Wilson, E., Hofer, L., Purtz, J., and Andersen, E.
Metabolic changes associated with transplantable ACTH-producing pituitary tumors of the mouse. Can. J. Biochem. Physiol. 35, 1057-65, 1957.

Serial No. NIAMD 8
1. Nutrition & Endocrinology
2. Endocrinology
3. Bethesda

N.I.A.

Project Title: [illegible]

Principal Investigator: Dr. Irvine Anderson, Dr. Robert H. Bates and Mrs. Frances Khanry

Man Years (Calendar Year 1958)
Total: 3
Professional: [illegible]
Other:

Project Description:

Objective: To investigate insulin in blood.

Methods and Major Findings: Methods have been developed for the extraction of insulin from plasma by Dr. Bates. The plasma solutions are being assayed for insulin activity by a method using the hypoglycemic method, alloxanized, diabetic rat.

By the Bates' method a precipitation of peripheral dog plasma, to which had been added I^{131} labelled insulin, a 74% recovery of the insulin by radioactive count was achieved and 83% by the method of bioassay. One explanation for the higher recovery by the bioassay method could be due to traces of unlabelled insulin in the peripheral blood, which is directly active but is not measured by radioactivity. The other explanation could be due to traces of unbound I^{131}.

By the bioassay method pancreatic vein blood of fasting cats and dogs has been found to contain approximately 65-75 microunits of insulin per ml. of plasma; if the blood sugar of the donor animal is raised to 300 mg. %, the insulin in the pancreatic vein is increased 5-10 fold. The conclusion is that glucose does stimulate the release of insulin.

Significance to NIAMD research: Studies of this nature should be of tremendous value in investigating diabetes.

Proposed course of project: Further studies are being carried out on the effect of glucose and amino acids on insulin secretion.

Part B not included.

Serial No. NIAMD 9
1. Nutrition and Endocrinology
2. Endocrinology
3. Methods

Part A.

Project Title: The influence of the central nervous system on metabolic functions -- Creatine and creatinine metabolism.

Principal Investigator: Dr. Kathryn Knowlton

Other Investigators: Mr. Leonard H. Hedda

Man Years: (Calendar Year 1958)
Total: 1 1/3
Professional: 1
Other: 1/3

Project description:

Objective: To distinguish between extrusion of intracellular creatine and failure of creatine incorporation by tissues as causes of creatinuria following transection of the spinal cord or other damage to the central nervous system.

Methods employed: The body creatine of a dog was labeled with N^{15} by feeding creatine synthesized to contain 60 atom % excess N^{15} in the glycine moiety. The spinal cord was then transected at C_7 level. From the urines collected starting at the time of N^{15} labeling, the excreted creatine and creatinine have been isolated, are being degraded to sarcosine and purified as the toluenesulfonyl derivative. The N^{15} content of these products will be determined by mass spectrometry.

Major findings: Techniques have been refined to allow isolation and purification of the toluenesulfonyl derivative of the sarcosine obtained from alkaline degradation of the comparatively small quantities of creatinine and creatine excreted daily by the dog. Work has begun on separation of the nitrogen on this derivative for determination of its N^{15} atom % excess.

Significance to NIAMD research: Since creatine phosphate occupies a place of some importance in intracellular energy interchanges of the mammalian body, knowledge of various phases of its metabolic mechanisms will contribute to understanding of diseased states. In

these particular studies, nerves of importance in neuroendocrine and neurometabolic problems should be more completely delineated.

Proposed course of project: When data from analysis for N^{15} abundance have been accumulated, interpretations will be made which dictate the course of further investigations. If the post-operatively excreted creatine contains the preponderance of N^{15}, the creatinuria must result from release by disintegrating muscle cells, or from failure of the biochemical mechanisms which normally retain creatine in the cell for its part in supporting energy transfer systems. If N^{15} does not appear in the postoperative creatine, the creatinuria must be due to failure of endogenously formed creatine to be incorporated into muscle cells and its consequent excretion in the urine.

Part B not included.

Individual Project Report
Calendar Year 1958

Serial No. NIAMD 10
1. Nutrition & Endocrinology
2. Endocrinology
3. Bethesda

Part A.

Project Title: Study of Anterior Pituitary Hormones: Isolation of Hormones.

Principal Investigators: Dr. Robert W. Bates and Dr. Peter G. Condliffe.

Other Investigators: Mr. Tulane B. Howard, Mrs. Mary M. Carrison, Dr. Jacob Furth, Dr. Richard Frape, Dr. Alexander Albert and Dr. Sidney Werner.

Cooperating Units: Harvard University, USNA (Beltsville), Mayo Clinic, and Columbia University.

Man Years (Calendar Year 1958):
Total: 3 2/3
Professional: 1 2/3
Other 2

Project Description:

Objectives: To isolate protein hormones, especially anterior pituitary hormones from glands; from functional, transplantable mouse pituitary tumors, and from blood; to study the primary and secondary structure of protein hormones, especially the anterior pituitary hormones; to investigate the nature of these hormones as they circulate in the bloodstream, to correlate chemical structure and biological function.

Methods employed: Hormones are extracted from pituitary glands, mouse pituitary tumors, and from blood. Crude extracts which may contain several hormones are fractionated by techniques commonly used in protein chemistry, such as salt precipitation, solvent fractionation, iso-electric precipitation, preparative electrophoresis, column chromatography and counter-current distribution. Physical properties of the purified hormones are determined by free electrophoresis, sedimentation, diffusion and other techniques for the study of proteins. Identification and quantitative estimation of the amino acid composition of the purified hormones are made by paper and ion exchange chromatography. Kinetic methods for the study of organic

reactions are used to prepare suitable derivatives of the hormones for structural analysis.

Major findings: Studies on isolation of protein hormones from glandular tissue and blood has been expanded this year to include prolactin, gonadotrophins and insulin, as well as thyrotrophin (TSH).

TSH: Procedural improvements have resulted in reduction of the time required for the chromatographic preparation of TSH and other hormones. About 100 mg. of potent TSH has been prepared by these methods and is now being used for physico-chemical studies.

It can be shown by chromatography that TSH activity is associated with at least 2 components that differ in net charge. When crude TSH is placed on carboxymethyl cellulose at pH 6, over 95% of the activity is adsorbed on the column. At pH 7 approximately 15% of the activity is unabsorbed. When rechromatographed at pH 7, this portion of the activity is again found in the unadsorbed fraction. However, it can be adsorbed at pH 6.

Exophthalmos producing substance (EPS), a common contaminant of TSH preparations, has been separated from TSH by chromatography on carboxymethylcellulose, being found in the acidic unadsorbed fraction at pH 6.

In collaboration with Dr. Sidney Werner we have tested a rabbit globulin fraction which was supposed to have anti-TSH activity. When injected into chicks it inhibited crude bovine TSH in the I^{131} depletion test. However, at the same dose level, it failed to inhibit either purified bovine TSH fractions obtained from chromatographic columns or from crude human TSH powder. These findings suggest that the chemical purity of the antigen is of utmost importance, if the antibody to be used for immunochemical assay is to be specific for a particular hormone.

Dinitrophenyl-threonine and DNP-phenylalanine are the principal DNP-amino acids found in hydrolysates of DNP-thyrotrophin suggesting that these 2 amino acids are the N-terminal groups of the hormone. An attempt to study the C-terminal residues was thwarted by lack of material, although in a single experiment it appeared that basic carboxypeptidase did not act on TSH.

Taller columns have been found to have no advantage over flat columns in percolation. When calcium chloride and ammonium acetate were used in place of sodium chloride during percolation similar results were obtained with respect to elution of TSH.

Prolactin: Three preparations of "pure" prolactin from different laboratories showed 3 components in starch gel electrophoresis, whereas they showed at least 2 major components during column chromatography.

A partial separation of these components present in potent prolactin preparations has been made and the separated components will be tested for their biological potency and their physicochemical differences will be investigated.

Prolactin is extractable in good yield (> 50%) by the percolation technique. It is eluted before any TSH in 76% ethanol medium when a 5% salt concentration is used. This concentration is not possible with NaCl, but is possible with ammonium acetate.

Gonadotrophin: A specimen of choriocarcinoma tissue was extracted by percolation. Its TSH content was so small that it could be accounted for by the TSH in the blood of the tissue. Since the gonadotrophin content of the tissue was high and probably singular in nature, bioassays for gonadotrophic activity were carried out which indicated yields of about 50% of the activity in the same fractions as the TSH (57% and 67% ethanol). The gonadotrophin present is probably similar to the so-called luteinizing hormone.

Insulin: Knowing the solubility of insulin, it was predicted that insulin should be present in the 76% ethanol-saline fractions obtained by percolation of blood plasma or pancreatic tissue. Dr. Anderson tested this fraction from human peripheral blood plasma for insulin and found 25 microunits/ml. of plasma.

Studies using I^{131} labeled insulin confirmed this finding. About 80% of the insulin was obtained in 2% by weight of the starting plasma. This 40 fold increase in concentration of insulin activity has made possible determination of the insulin activity in peripheral and pancreatic vein blood.

Significance to NIAMD research: The mechanism of action of pituitary hormones is not, as yet, understood. Progress will depend upon availability of pure hormones, a knowledge of their structure, and the accuracy and simplicity of the bioassay procedures. Progress in this area has been achieved by our improved methods for preparation and bioassay of the hormones. It is hoped that these methods will permit determination of blood levels of TSH in patients.

Proposed Course of Project: TSH is one of the few pituitary hormones which has not been isolated in pure form. Efforts to prepare pure TSH from various raw materials will continue and, when successful, full chemical and physical characterization will follow. The physicochemical homogeneity of TSH and prolactin will be assessed. Amino acid composition will be determined together with the terminal amino acids. For this purpose kinetic studies on enzymatic degradation and on the preparation of chemical derivatives will be carried out. Studies on the effect of TSH on thyroid physiology in the chick will continue. Time course studies will be made on the blood levels of TSH in radiothyroidectomized mice implanted with TSH producing tumors to determine when the blood levels of TSH rise. Determinations of the TSH level in blood of patients using our new extraction procedure will continue. Studies on blood content of prolactin and insulin are planned.

Part 3 included.

Part B

Publications other than abstracts from this project:

Cundliffe, P. C., Peter, R. W., and Krupp, E. M.
Fractionation of bovine thyrocrophin and luteinizing hormone on cellulose ion exchange columns. Biochimica et Biophysica Acta. (In Press)

Serial No. NIAMD 11
1. Nutrition & Endocrinology
2. Endocrinology
3. Metabolic

Part I.

Project Title: Studies on the secretion and metabolism of adrenocortical steroids in man and animals.

Principal Investigator: Dr. H. Wilson

Other Investigators: Dr. Seymour D. Lippett (NCI)
Dr. Lillian C. Butler (Georgetown)
Dr. David Y. Ryan

Cooperating Units: [illegible] University School of Medicine
[illegible] Cancer Institute

Man Years: (Calendar Year 195[illegible]
Total: 1.4
Professional: ...3
Other: 0.5

Project Description:

Objectives: The development of a simple technique for comprehensively surveying the entire pattern of urinary steroids has made possible the study of normal subjects with endocrine abnormalities. Two objectives have been pursued:

1. Determination of the normal and nature of the secretion of the normal human adrenal cortex during the complete absence of adrenocorticotropin (ACTH), as compared with that secreted during ACTH stimulation.

2. Establishment of the intermediate pathway by which dehydroepiandrosterone (DHA) is formed. The possibility being explored is that it originates from 5-17-hydroxy-pregnenolone. This abnormal intermediate is known to accumulate in many subjects with adrenal tumors, the adenomas having a very high DHA secretion. The normal adrenal cortex converts 5-pregnenolone to progesterone by means of the enzyme 3 β-dehydrogenase, and the next intermediate, 17-hydroxy-progesterone is the source of adrenal $C_{19}O_2$ steroids. Adrenal tumor tissue may have a relative insufficiency of 3 β-dehydrogenase, the resulting excess of 5-pregnenolone is converted to 17-hydroxy pregnenolone, which would yield DHA

rather than the normal $C_{19}O_2$ steroid, Δ^5-androstenediol. The same process, operating on a smaller scale, would account for the DHA in the urine of normal persons.

Methods employed:

1. Four female patients with mammary cancer, who were scheduled for surgical hypophysectomy, were followed for the first study. Urines were collected before hypophysectomy, and 3 or 4 weeks post-operatively during 24 hours of severe adrenal insufficiency following withdrawal of steroid therapy. Intravenous ACTH was then administered, and urines were analyzed at various intervals thereafter.

2. The urines of patients with adrenocortical tumors are being analyzed for the second study. Methods have been developed for the quantitative determination of Δ^5-17-hydroxy-pregnenolone, its chief metabolite Δ^5-pregnentriol, as well as for DHA. These are being applied to ascertain whether the two Δ^5-pregnene compounds are excreted in substantial amounts when DHA is elevated. Some subjects have been given injections of C^{14} cholesterol. It is planned to study the distribution of radioactivity among the above three urinary steroids at various times after the injection, enabling calculations of possible precursor: product relationships to be made.

The patients were under the supervision of Dr. M. B. Liggett of the NCI, who kindly made urine collections available to us.

Major Findings:

1. Steroid excretion with and without ACTH stimulation.

In the absence of ACTH, metabolites of cortisol showed by far the greatest diminution, reaching 8% of control levels. Androgen metabolites declined the least (to 30% control) and other types were intermediate. Several "abnormal" constituents, viz DHA and pregnenetriol, present in small amounts before hypophysectomy, were completely absent thereafter. However, certain other constituents are very little diminished in the absence of ACTH.

Quantitatively, the total measurable steroid excretion without ACTH varied from 3 to 6 mg. per 24 hours in the four subjects studied. One-fourth of this, or about 1 mg., were androgen metabolites, whereas metabolites of cortisol were about one-eighth the total, or 0.5 mg.

The effect of intravenous ACTH was reflected in the urine after a 12 hour refractory period. Hence, the adrenal cortex of these subjects remained capable of fairly prompt activation after about

½ weeks without ACTH.

Reversing the order of hypophysectomy, the first, and also the greatest effect of ACTH was to increase the output of cortisol metabolites. Thus, ACTH selectively increases the secretion of cortisol over and above that of other adrenocortical products.

These results provide the first accurate estimation of the total adrenocortical output of humans without ACTH, and show that the trophic hormone not only changes the amount of the steroid secretion, but has profound effects on its composition as well.

2. Preliminary results on five adrenal tumor patients showed that four had DHA excretion levels between 5 and 18 mgs. per day, or 10-20 times normal, and also excreted comparable amounts of pregnenetriol. One patient had quite a different pattern with only a slight elevation of DHA, and no Δ^5-pregnenetriol.

These findings lend credence to the hypothesized origin of DHA.

In addition to determinations of the three Δ^5 steroids, the patterns of other urinary steroid metabolites is being observed in these patients. Androgen metabolites have been uniformly elevated to 20-50 times normal levels. Cortisol metabolites may or may not be high. Those with increased cortisol production differedfrom the others in several other salient features as well. Two subjects appeared to excrete the abnormal "21-deoxy" steroid previously detected, which remains to be isolated and identified.

Significance to NIAMD Research: Elucidation of the general relations between adrenocortical activity and diverse states constitutes the long range objective and significance of these studies. To this end, the present results raise several contributions to our knowledge of adrenal steroid secretion and metabolism.

1. The study on hypophysectomized patients illuminates both the adrenocortical biosynthetic reaction sequence and the point or points of action of ACTH therein. Since it was found that ACTH selectively increases the secretion of cortisol over and above that of other adrenocortical products there are several possibilities regarding ACTH action:

a. ACTH may speed up 21- and 11-hydroxylation (in addition to its known effect on pregnenolone formation), thus, preferentially increasing the amount of cortisol synthesized.

b. The rate of formation of $C_{19}O_2$ androgens from 17-hydroxy-pregnenolone is not a direct function of the concentration of the latter, hence androgen formation is less directly affected by ACTH.

c. Adrenal androgens may be derived from cholesterol by a pathway not involving pregnenolone at all, which is less dependent on ACTH.

2. The studies on patients with adrenal tumors will give evidence as to whether DEA can be derived from a logical precursor Δ 5-17-hydroxy pregnenolone. At present the pathway by which DEA is formed in the adrenal cortex is not definitely known.

Various adrenal biosynthetic steps are greatly exaggerated in adrenocortical tumors. Other reactions are blocked, resulting in abnormal urinary metabolites which can be related to unusual intermediates. Our study of the urine steroid metabolite patterns of these subjects will expand our knowledge of these factors, yielding information which may be generally applicable.

Proposed course of project:

1. The study of hypophysectomized patients with and without ACTH is completed and now being written up for publication.

2. Analyses of these C_{19} steroids in the urine of patients with adrenal tumors will be carried out as described.

3. New collaborative studies with the NIAMD Clinical Endocrinological staff, concerning steroid patterns in adrenocortical disorders, is being initiated.

Part B included.

Part B

Publications other than contracts from this project:

Wilson, H., Burris, R., and Garrison, M.
Chromatographic procedure for the determination of urinary corticosteroids and C_{19} steroids. J. Clin. Endocrinol. & Metab. 18, 643, 1958.

Serial No. NIAMD 12
1. Nutrition & Endocrinology
2. Fractionation & Isola
3. Enzymes

Part A.

Project Title: large-scale processing of biological material.

Principal Investigator: John C. Krantz, Jr.

Other Investigators: None

Cooperating Units: NIH - Natural Products Laboratory

Man Years (Calendar Year 1958):

Total:	1.5
Professional:	0.50
Other:	1.00

Project Description:

Objectives: Many problems of importance in the biochemistry of disease require the isolation and identification of substances which are present in only trace amounts in the natural product. It becomes necessary in order to obtain sufficient quantities of the desired compound, to process large amounts of biological materials, such as liver, brains, excretory products, plant materials, etc.

Methods employed: The laboratory is equipped with large-scale apparatus, such as stills, filters, reactors and extraction bottles, etc. In most isolation problems the original small-scale process has to be modified and developed, so that it can be carried out efficiently on the large scale. This adaptation or process development is an important function of the laboratory. The degree of participation of the laboratory varies according to the needs of the specific problems.

Major findings: The laboratory facilities have been used in a variety of investigations carried on at NIH. Its main use has been in the preparation of concentrates from horse liver for prefolic acid, hog liver for converting enzymes, and the extraction of a great variety of plant materials for the Natural Products Laboratory (NPL). The fermentation unit has been used extensively for projects throughout NIH.

Significance to NIAMD research: Ordinary laboratories lack facilities to effectively carry out studies which require the extraction and processing of large quantities of biological materials. However, with the equipment and trained personnel of the large-scale laboratory, such investigations as the isolation of the prefolic acids can be undertaken and successfully completed.

Proposed course of project: To continue to offer assistance in large-scale operations as required by such projects as prefolic acid and the alkaloid problem (NMI).

Part B not included.

Part: B

Publications other than abstracts from this project:

Silverman, Milton, Gardiner, Rita C., and Cavalit, Paul T.
A method for the detection of N-formiminoglutamic acid in urine.
J. Natl. Cancer Inst. 20, 71-77, 1958.

Szulmajster, Jekisiel
Bacterial degradation of creatinine. II. Creatinine deiminase.
Biochim. Biophys. Acta 30, 154-163, 1958.

Silverman, Milton, and Pitney, Anna J.
Dietary methionine and the excretion of formiminoglutamic acid
by the rat. J. Biol. Chem. 233, 1179-1182, (1958).

[illegible]
Individual Project Report
Calendar Year 1958

Serial No. [illegible]
1. Nutrition & Endocrinology
2. Fractionation & Isolation
3. Bethesda

Part A.

Project Title: Microbiological assays for vitamins and amino acids

Principal Investigator: Milton Silverman

Other Investigators: E. Bakerman, M. Rendina

Cooperating units: None

Man Years (Calendar Year 1958):
Total: 3.5
Professional: 1.5
Other 2

Project Description:

Objective: The determination of various vitamins and amino acids in foodstuffs, body tissues and excretory products can be carried out by the use of microbiological assays. Samples submitted by investigators on nutritional and allied projects are analyzed for their content of the specific vitamin or amino acid under study.

Methods employed: The use of microbiological assays continues to be of major importance in the studies on folic acid. For example, with suitable test conditions one can distinguish between folic acid and its reduced derivatives. Further, the concentrations of 5- and 10-formyl derivatives of tetrahydrofolic acid in milli-microgram amounts can be determined in the presence of each other.

Major findings: In collaboration with Drs. O. Mickelsen and H. Windmueller (Virginia Polytechnical Institute), the reaction between the gaseous sterilizing agent, ethylene oxide, and the B vitamin, niacinamide, which results in the loss of the growth properties of the latter, has been defined. In the presence of ethylene oxide, niacinamide is converted into its N-2-hydroxyethyl derivative. The latter is devoid of growth activity for L. arabinosus and the chick and in itself does

not antagonize the growth promoting effects of nicotinic acid for these organisms. Preliminary evidence indicates that the inactivation of several other susceptible vitamins and amino acids is dependent on the alkylation of tertiary nitrogen, free amino or sulfide groups.

Significance to MIAMI research: These microbiological assay procedures are a very important tool for the measurement of the concentrations of various cell constituents, i. e., amino acids and vitamins.

Proposed course of project: To continue current microbiological problems and collaborate when called upon with other groups for projects requiring microbiological assays for vitamins and amino acids.

Part B included.

Part B:

Publications other than abstracts from this project:

Shay, A. E., Schreiber, W. E., Watkin, D. M., and Romine, M.
Effects of pantothenic acid deficient diets in patients with carcinomas. Cancer 11, 933-937, 1958.

Wind muller, R. C., Ackermann, C. F., Behrens, H., and Michelsen, O.
Reaction of ethylene oxide with nicotinamide and nicotinic acid.
J. Biol. Chem. (Accepted for publication)

NIH - NIH
Individual Project Report
Calendar Year 1958

NIAMD 14
1. Nutrition & Endocrinology
2. Fractionation & Isolation
3. Methods

Part A.

Project Title: Studies on Folic Acid

Principal Investigators: M. Silverman and J. C. Rabinowitz

Other Investigators: R. Gardiner, K. O. Donaldson
J. Szulmajster (until September)
R. Kisliuk (since September)

Cooperating Units: None

Man Years (Calendar Year 1958):
Total: 5
Professional: 3
Other: 2

Project Description:

Studies on folic acid:
(a) Naturally-occurring forms of (pre)folic.
(b) Enzymatic conversion of prefolic acid to known forms.
(c) Delineation of the reaction sequence (in the rat) occurring in the conversion of formimino-carbon (from formiminoglutamic) to the methyl level.
(d) Elucidation of the pathway of the synthesis of methionine in Escherichia coli.
(e) Separation and purification of the enzymes and vitamin B_{12} co-factor from E. coli, which are involved in the synthesis of methionine from homocysteine and one carbon donors.

Major findings: The enzymatic system which converts prefolic acid A to citrovorum factor has been found to consist of two parts, one, conversion to tetrahydrofolic acid or a product very similar to it, and, two, formylation to citrovorum factor. The first reaction has been studied intensively and flavin adenine dinucleotide was found to actively participate in this conversion. Whether its role is catalytic is still

under study. The enzyme concerned in this reaction was partially purified (10 fold over that of water extract of liver).

It has been shown that the rat which is fed a diet low in protein (9% casein) and high in fat (20% Crisco) will, in the absence of dietary folic acid or vitamin B_{12}, excrete formiminoglutamic acid. The excretion of the formimino compound is reduced by dietary methionine or homocysteine. It appears that a major pathway for the formimino compound in such rats involves the conversion of the available 1-carbon unit to a methyl group, a reaction sequence involving both folic acid and vitamin B_{12}. This type of rat which shows a requirement for both folic acid and vitamin B_{12} in the metabolism of formiminoglutamate should prove a useful tool in studies involving the biochemical relationship of these two vitamins.

Significance to WAAD research: The isolation and identification of the various metabolites of folic acid will result in a better understanding of the biochemistry of the anemias and leukemias. The mechanisms by which folic acid derivatives influence the formation of purines are being widely studied. This project is directed at discovering how the various forms of folic acid exist in the body and how they are changed from inactive to active forms.

Proposed course of project:

(1) To isolate the two forms of prefolic acid and establish their chemical structure.
(2) To study the reactions concerned in the transformation of prefolic acid to citrovorum factor and to purify the enzyme.
(3) To attempt to establish, at the biochemical level, the inter-relationship of folic acid and vitamin B_{12}.

Part B included.

NATIONAL INSTITUTES OF ARTHRITIS AND METABOLIC DISEASES

ANNUAL PROJECT REPORT

Calendar Year 1958

Summary Sheet

LABORATORY OF BIOCHEMISTRY AND METABOLISM

Estimated Obligations for FY 1959

Total - $750,454
Direct - $457,000
Reimbursement: - $293,454

Project number 15 thru 32 included

Individual Project Report
Calendar Year 1958

Serial No. NIAMD- 15
1. Biochemistry & Metabolism
2. Enzymes & Cellular Biochemistry
3. Bethesda

Part A.

Project Title: Carbohydrate Metabolism

Principal Investigator: Dr. Gilbert Ashwell

Other Investigators: Dr. Jean Hickman

Cooperating Units: Dr. J. D. Smiley, Research Associate
Dr. H. A. Cynkin, Visiting Scientist
Mr. Albert Wahba, Damon Runyon Fellow
Dr. J. J. Burns, National Heart Institute, Laboratory of Chemical Pharmacology

Man Years (calendar year 1958):
Total: 5
Professional: 4
Other: 1

Project Description:

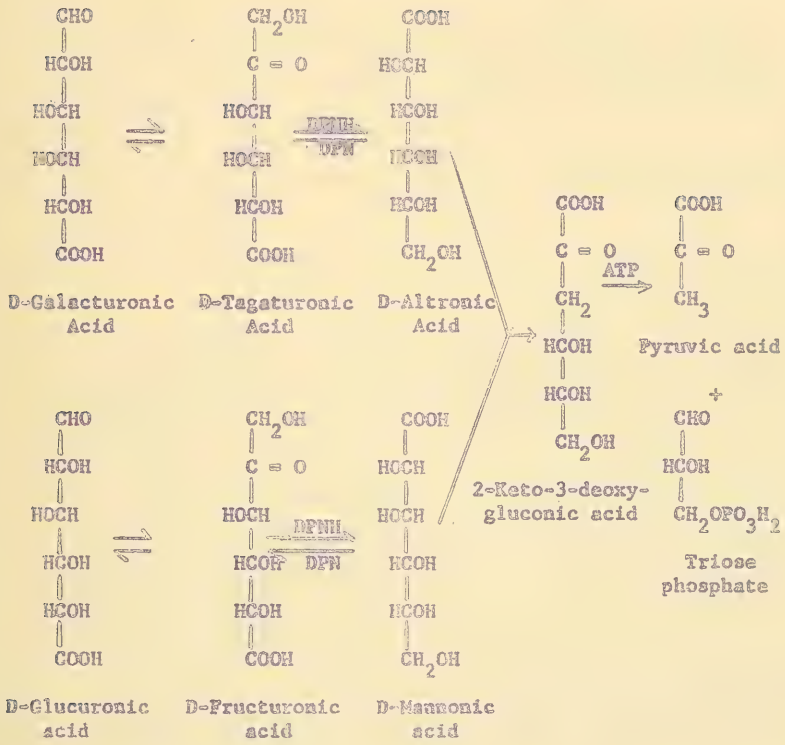
Objectives: An investigation of uronic acid metabolism in mammalian and bacterial systems was undertaken in an attempt to elucidate the biosynthesis of L-ascorbic acid and L-xylulose, both of which are known to arise from glucuronic acid.

Methods Employed: In general, the methods employed involve an enzymatic or colorimetric determination of substrate utilization or product formation. This is usually followed by purification of the enzyme system involved. Isolation of the reaction products is attempted by use of paper and column chromatography and final identification made by preparation of the appropriate crystalline derivatives.

Major Findings: In the last annual report, it was stated that cell free preparations of E. coli had been obtained which were capable of degrading both galacturonic and glucuronic acids by a novel mechanism not involving the formation of free pentoses. Further work has resolved this problem and the following reaction sequence has been demonstrable:

Part B included X Yes ___ No

Major Findings, cont.



It is interesting to note that of the five intermediates in this pathway, only 2-keto-3-deoxy gluconic acid had previously been known to be a biologically active compound.

In a continuing collaboration with Dr. John Burns and Mr. Julian Kanfer of the Heart Institute, considerable progress has been made in the elaboration of the biosynthetic pattern of L-ascorbic acid and L-xylulose. Both of these compounds were known to arise from L-gulonic acid although no information was available as to the mechanism of the reaction. It has now been demonstrated that 3-keto-L-gulonic acid is the intermediate in the conversion of L-gulonic acid to L-xylulose and that this compound is not the precursor of L-ascorbic acid. Similarly,

Major Findings, cont.

on the basis of studies with model compounds, 2-keto-L-gulano lactone appears to be active intermediate in the ascorbic acid synthesizing system and to play no role in the formation of L-xylulose. Consequently, the following reaction sequence has been proposed:

Further studies on the metabolism of L-ascorbic acid by the rat kidney have very recently lead to the tentative identification of a new and provocative compound, L-lyxonic acid. No further information is as yet available on the mechanism or significance of this finding.

Significance to NIAMD Research: It is expected that a study of these compounds and the pathways by which they are utilized will contribute to our knowledge of the over-all metabolic process and thus lead to a more rational approach to the understanding and treatment of the pathological alterations in metabolism.

Proposed Course of Project: A detailed study of the newly discovered enzymatic reactions of the uronic acid pathway is in progress. It is hoped that the information obtained in these studies will provide a better understanding of the lesser known mechanisms of carbohydrate metabolism. Particular emphasis will be placed on the reactions of 2-keto-3-deoxy gluconic acid as a potential source of 2-deoxyribose. In addition, the implications inherent in the finding of L-lyxonic acid as a product of mammalian metabolism will be explored in the attempt to determine the significance of this compound in Vitamin C deficiency.

Part B.

Publications during 1958:

Hickman, J. and Ashwell, G., Purification and Properties of D-Xylulokinase in Liver, J. Biol. Chem., 232, 737 (1958).

Wahba, A., Hickman, J., and Ashwell, G., Enzymatic Formation of D-Tagaturonic and D-Fructuronic Acid, J. Am. Chem. Soc., 80, 2594 (1958).

Ashwell, G., Wahba, A., and Hickman, J., A New Pathway of Uronic Acid Metabolism, Biochim. Biophys. Acta, 30, 186 (1958).

Burns, J. J. and Ashwell, G., L-Ascorbic Acid, The Enzymes, edited by Lardy, et al, Academic Press, in press.

Ashwell, G., Kanfer, J., and Burns, J. J., Studies on the Mechanism of L-Xylulose Formation by Kidney Enzymes, J. Biol. Chem., in press.

Kanfer, J., Burns, J. J., and Ashwell, G., L-Ascorbic Acid Synthesis in a Soluble Enzyme System from Rat Liver Microsomes, Biochim. Biophys. Acta, in press.

Hickman, J. and Ashwell, G., A Sensitive and Stereospecific Enzymatic Assay for Xylulose, J. Biol. Chem., in press.

PHS-NIH
Individual Project Report
Calendar Year 1958

Serial No. NIAMD- 16
1. Biochemistry & Metabolism
2. Enzymes & Cellular Biochemistry
3. Bethesda

Part A.

Project Title: Studies on the Structure, Biosynthesis and Intermediary Metabolism of Nucleic Acids and Nucleotides.

Principal Investigator: Dr. Leon A. Heppel

Other Investigators: Dr. Russell J. Hilmoe
Dr. Maxine F. Singer
Dr. Marianne Grunberg-Manago
Dr. G. H. Khorana

Cooperating Units: W. E. Razzell, Jr., British Columbia Research Council (2 weeks)

Man Years (calendar year 1958):
Total: 4 years 1 month
Professional: 3 years 3 months
Other: 5/6 year

Project Description:

Objectives: The object of this project is to discover pathways for the biosynthesis and breakdown of nucleic acids and smaller polynucleotides.

Methods Employed: Biosynthesis and degradation of RNA and other polynucleotides is studied, using enzymes purified from bacterial, plant and animal sources. The reactions are followed by means of paper and column chromatography, chemical analysis and isotope tracer methods. Also, purified enzymes are used as specific analytical reagents for study of polynucleotide structure.

Major Findings: Highly purified bacterial polynucleotide phosphorylase forms polymers from nucleoside diphosphates only after a prolonged lag period. As reported last year, oligonucleotides such as pApA overcome the lag. Primers of this type are incorporated into the newly synthesized polymer,

Part B included X Yes ___ No

Major Findings, cont.

forming the beginning portion of the new chain.

It has now been shown that oligonucleotides of quite another kind are equally effective in overcoming the lag period and stimulating polymerization. These are compounds such as the dinucleotide ApUp, characterized by a 3'-phosphomonoester endgroup. Such compounds cannot be incorporated into polymer and they are recovered unchanged at the end of the reaction, yet by their presence it is possible for polymerization to occur. This result is considered to be highly significant, for it shows that formation of polynucleotides such as RNA can be stimulated by a primer which need not be incorporated and therefore could act as a template. Materials such as RNA and poly A also act as primers and are probably not incorporated, but with these large molecules it is difficult to prove their point.

An exception to all of this is found when enzymatic polymerization is attempted using only guanosine diphosphate (GDP). The synthesis of polyguanylic acid has never been accomplished previously, despite attempts in many laboratories. It has now been found that GDP can be polymerized, but only in the presence of primers of the type which can be incorporated. Thus:

$$pApA + n\ GDP \rightleftharpoons pApApGpG \ldots\ldots pGpGpG \qquad (1)$$

The enzyme catalyses addition of guanylic acid residues to the adenosine containing dinucleotide, pApA, to form polymer.

A new activity has been discovered for polynucleotide phosphorylase, namely transnucleotidation, or the transfer of nucleotide residues from one polynucleotide to another. Extensive studies have also been carried out on the mechanism of the exchange of P^{32} labeled inorganic phosphate and nucleoside diphosphates, and on factors which influence the rate of phosphorolysis. An interesting finding is that phosphorolytic breakdown of polynucleotides occurs in stepwise fashion, starting from the nucleoside end of the molecule the right hand end of the polymer represented in equation (11). Further, it has been found that hydrolysis of polynucleotides by snake venom phosphodiesterase occurs in precisely the same stepwise fashion.

Studies of nucleases have been continued. The liver nuclei enzyme which hydrolyses poly A to small oligonucleotides

Major Findings, cont.

has been solubilized and partially purified. Methods for separation of the products on Ecteola columns have been worked out. The action of ryegrass ribonuclease has been studied; it was found to split every diester bond in polynucleotides, regardless of endgroup structure.

Significance to NIAMD Research: The present studies help to clarify mechanisms for RNA synthesis and degradation. Consequently they are of significance for problems of hereditary mechanisms, certain metabolic diseases, and plant and animal viruses. Governing principles found in the action of polynucleotide phosphorylase probably also hold for RNA synthesis, for the two systems are becoming increasingly similar as investigation proceeds. Finally, results of the present work are important to studies of protein biosynthesis, (1) in the analysis of structure of S-RNA and (2) in proving that activated amino acids go to the ends of S-RNA chains.

Proposed Course of Project: Further studies on mechanisms of RNA biosynthesis and nuclease specificities are contemplated. The transnucleotidation reaction catalyzed by polynucleotide phosphorylase will be examined further. Dr. Singer proposes to use this and other enzymes to investigate the structure of S-RNA, the material which accepts activated amino acids and transfers them to form microsomal protein.

Part B.

Publications during 1958:

Strominger, J. L., Heppel, L. A., and Maxwell, E. S., Nucleoside Monophosphate Kinases I. Transphosphorylation Between Adenosine Triphosphate and Nucleoside Monophosphates, Biochim. et Biophys. Acta, in press.

Heppel, L. A., Strominger, J. L., and Maxwell, E. S., Nucleoside Monophosphate Kinases II. Transphosphorylation between Adenosine Monophosphate and Nucleoside Triphosphates, Biochim. et Biophys. Acta, in press.

Shuster, L., Khorana, H. G., and Heppel, L. A., The Mode of Action of Ryegrass Ribonuclease, Biochim. et Biophys. Acta, in press.

Heppel, L. A., and Rabinowitz, J. L., Enzymology of Nucleic Acids, Purines and Pyrimidines, Ann. Reviews of Biochem., 27, 613 (1958).

Singer, M. F., Phosphorolysis of Oligonucleotides by Polynucleotide Phosphorylase, J. Biol. Chem., 232, 211-227 (1958).

Heppel, L. A., Singer, M. F., and Hilmoe, R. J., Mechanism of Action of Polynucleotide Phosphorylase, Proc. of the New York Academy of Sciences, in press.

Hilmoe, R. J., The Effect of Endgroups and The Initial Site of Attack on Polynucleotides by Polynucleotide Phosphorylase and Certain Phosphodiesterases, Proc. of the New York Academy of Sciences, in press.

Singer, M. F., Heppel, L. A., Hilmoe, R. J., Ochoa, S. and Mii, S., Enzymatic Synthesis of Polyribonucleotides, Symposium of the Third Canadian Cancer Research Conference, in press.

Individual Project Report
Calendar Year 1958

Serial No. NIAMD- 17
1. Biochemistry & Metabolism
2. Enzymes & Cellular Biochemistry
3. Bethesda

Part A.

Project Title: Enzymatic Utilization of Model Compounds

Principal Investigator: William B. Jakoby

Other Investigators: Edward M. Scott

Cooperating Units: Dr. [illegible] V. Tomeo, Fellow of the Jane Coffin Childs Memorial Fund for Medical Research
Dr. M. Hirenberg, Fellow of the American Cancer Society (Project Number)

Man Years (calendar year 1958):
Total: 3-5/6
Professional: 2
Other: 1-5/6

Project Description:

Objectives: This work is concerned with the investigation of reactivity of various chemical groupings in enzyme catalyzed reactions in the belief that such enzyme studies are basic to our understanding of biological phenomena. Simple model compounds, each containing the desired chemical groupings, are used as substrates.

Methods Employed: By use of the enrichment culture technique, microorganisms were obtained with the ability to grow on various model compounds as sole carbon source. Enzymes from these organisms were investigated by the usual techniques.

Major Findings: Among the first group of compounds selected for study were certain simple derivatives of acetylene. It was found that the initial step in the metabolism of acetylene mono- and dicarboxylic acids involves hydration with the formation of malonic semialdehyde and pyruvic acid, respectively. While these specific compounds are not likely to be found in nature, a vast number of acetylene derivatives including compounds with antibiotic activity, do occur and the probability of their utilization by similar mechanisms is great.

Part B included X Yes ____ No

Major Findings, cont.

A study of the metabolism of the nitrogen containing heterocycle, pyrrolidine, in a bacterial system has indicated the following sequence of intermediates: pyrrolidine ⟶ Δ^1-pyrroline ⟶ γ-aminobutyraldehyde ⟶ γ-aminobutyric acid ⟶ succinic semialdehyde ⟶ succinic acid. This investigation has led to the description of a system effective in the transamination and subsequent oxidation of γ-aminobutyric acid, an important reaction sequence of brain. These enzymes have been extensively purified and an investigation of their kinetics has allowed the formulation of probable reaction mechanisms. The transaminase, for example, functions in such a manner so that either the amino acid or the α-ketoacid, but not both, are on the holoenzyme at any one time. In contrast to this mechanism, succinic semialdehyde oxidation requires the presence of both aldehyde and pyridine nucleotide on the enzyme.

It is also of interest that putrescine, formed in the gastrointestinal tract of mammals, can be metabolized by bacterial enzymes in a pattern similar to that used by pyrrolidine, namely, putrescine ⟶ γ-aminobutyraldehyde ⟶ γ-aminobutyric acid ⟶ succinic semialdehyde ⟶ succinic acid.

Significance to NIAMD Research: Each of the compounds studied impinges on some area of vital interest in the fields of biology and medicine. The information obtained by studies such as these form the basis of application to more practical fields. In this connection should be mentioned the metabolism of the acetylenic antibiotics as well as of γ-aminobutyric acid utilization in brain.

Proposed Course of Project: It is expected that the enzymes involved in γ-aminobutyric acid formation will be investigated in both the microbial system as well as in brain. The metabolism of other model compounds will be continued.

Part B.

Publications during 1958:

Yamada, E. W., and Jakoby, W. B., Enzymatic Utilization of an Acetylenic Compound, J. Am. Chem. Soc., 80, 2343 (1958).

Yamada, E. W., and Jakoby, W. B., Enzymatic Utilization of Acetylenic Compounds I. An Enzyme Converting Acetylenedicarboxylic Acid to Pyruvate, J. Biol. Chem., 233, 704 (1958).

Jakoby, W. B., Aldehyde Oxidation I. Dehydrogenase from Pseudomonas fluorescens, J. Biol. Chem., 232, 75 (1958).

Jakoby, W. B., Aldehyde Oxidation II. Evidence for Closely Juxtaposed Sulfhydryl Groups on Dehydrogenases, J. Biol. Chem. 232, 89 (1958).

Jakoby, W. B., and Bhat, J. V., Microbial Metabolism of Oxalic Acid, Bact. Rev., 22, 75 (1958).

Scott, E. M., and Jakoby, W. B., Pyrrolidine Metabolism: Soluble γ-aminobutyric Transaminase and Semialdehyde Dehydrogenase, Science, 128, 361 (1958).

Jakoby, R. K., and Jakoby, W. B., Lactic Dehydrogenase of Cerebrospinal Fluid in the Differential Diagnosis of Cerebrovascular Disease and Brain Tumor, J. Neurosurgery, 15, 45 (1958).

Scott, E. M., and Jakoby, W. B., Soluble γ-aminobutyric-glutamic Transaminase from Pseudomonas fluorescens, J. Biol. Chem., in press.

Jakoby, W. B., and Scott, E. M., Aldehyde Oxidation III. Succinic Semialdehyde Dehydrogenase, J. Biol. Chem., in press.

Yamada, E. W., and Jakoby, W. B., Enzymatic Utilization of Acetylenic Compounds II. Acetylenemonocarboxylic Acid Hydrase, J. Biol. Chem., in press.

PHS-NIH
Individual Project Report
Calendar Year 1958

Serial No. NIAMD- 18
1. Biochemistry & Metabolism
2. Enzymes & Cellular Biochemistry
3. Bethesda

Part A.

Project Title: The Enzymatic Transformation of Carbohydrates and their Intermediary Metabolism

Principal Investigator: Dr. B. L. Horecker

Other Investigators: Mrs. Ann Ginsburg
Mr. William Pricer
Dr. Sandro Pontremoli

Cooperating Units: None

Man Years (calendar year 1958):
Total: 3-5/6
Professional: 2-1/3
Other: 1-1/2

Project Description:

Objectives: To study the pathways of carbohydrate metabolism and their relation to the synthesis of cellular components and to cell growth and function. This year the particular objectives were (1) to provide information on the metabolism of deoxyribose and (2) to study the mechanisms of carbohydrate transport.

Methods Employed: The pathway of deoxyribose fermentation was studied in a microorganism capable of fermenting this sugar. Enzyme preparations from this organism were purified and used for the synthesis of intermediates. Carbohydrate transport mechanisms are studied with living bacteria, using kinetic methods and suitable mutants which lack the capacity to metabolize the sugars examined.

Major Findings: The study of deoxyribose metabolism in Lactobacillus plantarum, initiated in the preceding year, was continued. The adaptive deoxyribose kinase, which catalyzes the formation of deoxyribose-5-phosphate was purified and shown to react with D-ribose, in addition to dioxyribose. It differs, however, from the ribokinase formed when this organism is grown on ribose, since the

Part B included X Yes ____ No

Major Findings, cont.

latter enzyme is inactive with deoxyribose. The reaction product was isolated and identified as deoxyribose-5-phosphate. With the isolation of this enzyme, all of the steps in deoxyribose fermentation in L. plantarum have been characterized.

Further purification of the deoxyribose aldolase has been accomplished. This enzyme assumes added importance in view of the isotope data obtained during the previous year which suggest that some synthesis of deoxyribose in animal cells may occur by a C_2, C_3 condensation. The enzyme, purified about 100 fold, has been found to have a reasonable affinity for acetaldehyde, with a dissociation constant of about 10^{-3}M, compared with a dissociation constant 5×10^{-4}M for deoxyribose. This removes the major objection to acetaldehyde as a precursor of deoxyribose phosphate

Studies on the physiological role of transketolase and transaldolase in pentose synthesis in animal tissues are in progress. Both of these enzymes require acceptors for action, yet in liver supernatant fractions synthesis of ribose phosphate from hexose monophosphate occurs in the absence of added acceptors. This has also been found to be true for mixtures of the purified enzymes. It is possible that the enzymes can act in concert, each providing the triose phosphate or tetrose phosphate acceptor needed for the other. This would be a new pattern of enzyme action and is now under investigation.

In the studies on carbohydrate transport, emphasis has been placed on a newly discovered system for the transport of galactose in E. coli. This organism, which lacks galactokinase, cannot metabolize galactose but nevertheless concentrates this sugar within the cells. This suggests that phosphorylation is not essential to transport. The galactose transport mechanism is highly specific and no other substrates have been found. It is not identical with the system which catalyzes the transport of galactosides or of glucose. It requires energy and is poisoned by dinitrophenol. Further work will be directed toward an elucidation of the biochemical mechanism of carbohydrate transport.

Significance of This Research: The biological origin of deoxyribose remains one of the important unsolved problems in carbohydrate metabolism. It is of particular importance since this sugar is a component of deoxyribonucleic acid and therefore essential for cell growth and division. With respect to the transport mechanisms, these are becoming increasingly implicated in cellular control mechanisms, as in the mode of action of insulin, and a better understanding of their chemical nature is of the greatest importance.

Proposed Course of Project: Further efforts will be made to determine the mechanism of deoxyribose synthesis in animal cells and bacteria. The transport mechanisms will be examined in an effort to find covalent compounds between the substrates involved and cellular membrane components and the mechanism of substrate activation for transport will be studied.

Part B.

Publications During 1958:

Horecker, B. L. and Hiatt, H. H., Pathways of Carbohydrate Metabolism in Normal and Neoplastic Cells, The New England J. of Med., 258, 177-184, 225-232 (1958).

Hiatt, H. H., Goldstein, M., Lareau, J., and Horecker, B. L., Pathway of Hexose Synthesis from Pyruvates in Muscle, J. Biol. Chem., 232, 303 (1958).

Heath, E. C., Hurwitz, J., Horecker, B. L., and Ginsburg, A., Pentose Fermentation by Lactobacillus Plantarum I. The Cleavage of Xylulose-4-phosphate by Phosphoketolase, J. Biol. Chem., 231, 1009 (1958).

Heath, E. C., Horecker, B. L., Smyrniotis, P. S., and Takagi, Y., Pentose Fermentation by Lactobacillus Plantarum II. L. Arabinose Isomerase, J. Biol. Chem., 231, 1031 (1958).

Burma, D. P. and Horecker, B. L., Pentose Fermentation by Lactobacillus Plantarum III. Ribulokinase, J. Biol. Chem. 231, 1039 (1958).

Burma, D. P. and Horecker, B. L., Pentose Fermentation by Lactobacillus Plantarum IV. L-Ribulose 5-Phosphate 4-Epimerase, J. Biol. Chem., 231, 1053 (1958).

Domagk, G. F. and Horecker, B. L., Pentose Fermentation by Lactobacillus Plantarum V. Fermentation of 2-Deoxy-D-Ribose, J. Biol. Chem., 233, 283 (1958).

Horecker, B. L., Le Cycle des Pentoses et sa Signification Physiologique, Bull. Soc. Chim. Biol., 40, 565 (1958).

Honors and Awards: Dr. Horecker addressed the first meeting of the Swiss Biochemical Society and was elected the first foreign member of the Society.

Dr. Horecker addressed the Societe' de Chimie Biologique and was awarded the Society Medal.

Individual Project Report
Calendar Year 1958

Serial No. NIAMD- 19
1. Biochemistry & Metabolism
2. Enzymes & Cellular Biochemistry
3. Bethesda

Part A.

Project Title: a. Studies on Amino Acid Incorporation in Normal and Viral Infected Bacteria

b. Structure Studies of Certain Cyclitols

Principal Investigator: Arthur Weissbach

Other Investigators: None.

Cooperating Units: None.

Man Years: (Calendar year 1958)

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives: The purpose of these investigations is to a. determine how viral infection affects the cell's ability to incorporate amino acids into protein, b. to obtain more evidence for the stereochemical form of scyllitol diborate.

Major Findings: a. The infection of E. coli Bacteria with a lethal virus (T_2) causes a marked change in the metabolic activity of the bacterial cell. One of the most dramatic changes occurs in the type of proteins synthesized since new bacteriophage protein must be formed. The effect of viral infection upon the amino acid incorporating system of E. coli is now being studied. Kinetic experiments have indicated that subcellular particles obtained from phage infected E. coli (in vivo experiments) seem to incorporate much less amino acid than do the normal cells under the same conditions. These studies were carried out using rapidly growing cells and separating the proteins, obtained from these cells, on Sober-Peterson exchange columns.

Protein synthesis is assumed by many workers to proceed,

Part B included X Yes ___ No

Major Findings, cont.

in general, in the following steps:

1. Amino Acid + ATP ⟹ "ACTIVATED" amino acid

2. "ACTIVATED" amino acid + Soluble RNA ⟹ Soluble RNA - Amino Acid

3. Soluble RNA - Amino Acid + microsome ⟹ microsomes - Amino Acid

Studies of these steps in the viral infected cell in vitro are now being done. Results have shown that there is no inhibition of the normal amino-acid activating system EQUATION (1) nor of the transfer of the activated amino acid to Soluble RNA EQUATION (2) in the infected cell in the latent period. Furthermore, the soluble RNA isolated from the phage infected cell seems to accept amino acids in this system as well as does soluble RNA isolated from normal uninfected cells. However, studies on the RNA synthesizing system, polynucleotide phosphorylase, will be carried out in both the infected and uninfected cells. Studies on the direct ribosomal incorporation of amino acids in vitro are now in process.

b. Previous annual reports have described the isolation of Scyllitol Diborate, a cyclitol derivative, which seems to present the unique feature of having all its hydroxyl groups bound in an axial conformation. Various physical and chemical methods are being employed to prove the steric chemistry of this compound. At present, the nuclear magnetic resonance spectra is being obtained and may supply the final proof of structure. Other methods used in these studies have been Infra-red spectra, acetylation procedures, etc.

Part B.

Publications during 1958:

Weissbach, A., Acyclitol Diborate, J. Org. Chem., 23, 39[illegible] (1958).

Ginsburg, V., Weissbach, A., and Maxwell, E., Formation of Glucuronic Acid from Uridinediphosphate Glucuronic Acid, Biochim. Biophys. Acta, 28, 649 (1958).

Weissbach, A. and Hurwitz, J., The Formation of 2-keto-3-deoxy Heptonic Acid in Extracts of E. coli I. Isolation and Characterization, J. Biol. Chem., in press.

Hurwitz, J. and Weissbach, A., The Formation of 2-keto-3-deoxy Heptonic Acid in Extracts of E. coli II. Enzymatic Studies, J. Biol. Chem., in press.

Serial No. NIA
1. Biochemistry & Nutrition
2. Intermediary Metabolism
3. Bethesda

PHS-NIH
Individual Project Report
Calender Year 1958

Part A.

Project Title: The Mechanism of Action of Enzymes and Hormones

Principal Investigators: Dr. Y. J. Topper, Dr. L. A. Pesch and Dr. E. R. Simon

Other Investigators: Dr. B. Bloom and Dr. S. Segal

Cooperating Units: Clinical Endocrinology Branch, NIAMD (Grace #131.)

Man Years (calendar year 1958):
Total: 4-2/3
Professional: 3-2/3
Other: 1

Project Description:

Objectives - The immediate objectives of this project are to ... elucidate the mode of action of aldolase and triose phosphate isomerase and to study the effect of various steroids on the metabolism of D-galactose.

Methods Employed - In the enzyme mechanism studies complex formation between substrate and enzyme is followed spectrophotometrically.

The effect of hormones on D-galactose metabolism is studied *in vitro* and *in vivo* by measuring $^{14}CO_2$ production from isotopic galactose.

Major Findings - Since the effect of steroids on D-galactose metabolism was reported last year the following observations have been made. (1) The only tissue other than liver which responds in this respect is small intestine. (2) Of the steroids tested progesterone, testosterone and androsterone exert equal and maximal effects. 3) A broken cell system has been obtained which manifests the hormone effect. (4) The onset of cataracts in rats maintained on a high galactose diet is delayed when the animals are treated with progesterone. (5) Progesterone treatment endowed a galactosemic subject with some ability to convert D-galactose to CO_2.

Significance to NIAMD Research - Studies on hormonal control of sugar catabolism might be expected to shed some light on certain metabolic diseases.

Proposed Course of Project - 1. We will attempt to obtain information relating to the "active site" of aldolase. 2. The precise site of action of progesterone on galactose oxidation will be sought.

Part B included: Yes

PHS-1MH
Individual Project Report
Calendar Year 1958

Part B. Honors, Awards and Publications

Publications other than abstracts from this project:

Pesch, L. A. and Popper, Y. J. Effects of steroids upon galactose oxidation in vitro. Biochim. Biophys. Acta 30, 206-207 (1958).

Honors and Awards relating to this project: None.

Serial No. NIAMD- 21
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: The Binding of Purines and Pyrimidines and Their Derivatives to Purified Protein

Principal Investigators: Dr. J. H. Bryant and Dr. F. Tietze

Other Investigator: Dr. D. Stetten, Jr.

Cooperating Units: None

Man Years (calendar year 1958):
Total: 1-2/3
Professional: 1
Other: 2/3

Project Description:

Objectives - To compare the degree of binding between purified protein and the various purines, pyrimidines and their corresponding nucleosides and nucleotides, and to determine if there is a significant difference between the binding to protein of those components of RNA as compared with those of DNA.

Methods Employed - The dialysis method of Klotz is being used to determine the degree of binding. Crystalline bovine serum albumin is used as the purified protein.

Major Findings - In compounds studied thus far, the degree of binding has corresponded with the net charge on the molecule in all cases except that of thymidylic acid in which the binding is significantly less than in other nucleotides.

Significance to NIAMD Research - Whether protein synthesis occurs on an RNA template remains to be proven. Some support for this concept might be obtained from experiments demonstrating a lesser degree of binding between DNA derivatives and protein than between RNA derivatives and protein.

Proposed Course of Project - More compounds will be studied in order to extend preliminary observations.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1958

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Bryant, J. H., Leder, I. G. and Stetten, D., Jr. The release of chondroitin sulfate from rabbit cartilage following the intravenous injection of crude papain. Arch. Biochem. Biophys. 76, 122-130 (1958).

Spicer, S. S. and Bryant, J. H. Systemic effects in rabbits receiving injections of papain and chondroitin sulfate. Am. J. Path. 34, 61-75 (1958).

Honors and Awards relating to this project: None

Se[illegible]
1. Biochemistry & Metab[illegible]
2. Intermediary Metabolism
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: The Biosynthesis of Thiamine

Principal Investigator: Dr. I. G. Leder

Other Investigators: None

Cooperating Units: None

Man Years (calendar year 1958):
Total: 2
Professional: 1
Other: 1

Project Description:

Objectives - To study the mechanism of synthesis of the vitamin thiamine.

Methods Employed - The enzymes which catalyze sequential steps in the synthesis of thiamine will be isolated from crude extracts of bakers' yeast by classical fractionation procedures and by column adsorption and elution techniques. The enzymatically synthesized vitamin and precursor compounds will be tested biologically with various mutants of neurospora and E. coli. Intermediates formed by purified enzyme systems will be isolated and studied by chromatographic, electrophoretic and spectrophotometric techniques.

Major Findings - Crude bakers' yeast extracts have been found to catalyze a condensation of 2-methyl 4-amino 5-hydroxymethyl pyrimidine or the corresponding 5-methyl phosphate, with 4-methyl 5-(β-hydroxyethyl) thiazole to form thiamine. Two protein fractions have been separated from such extracts, neither of which can form thiamine alone, but which do so when combined. The first of these fractions activates the pyrimidine and the second catalyzes the condensation with the thiazole.

Significance to NIAMD Research - Cyclic compounds containing sulfur are represented by such diverse compounds as biotin, penicillin and thiamine. It is hoped that this study will contribute to our understanding of the synthesis and metabolism of compounds of nutritional and medicinal importance in man and in microorganisms.

Proposed Course of Project - The enzymes revealed in initial studies will be purified and the activated pyrimidine isolated and characterized. With the aid of the purified enzymes, extracts of mutant microorganisms and of mammalian liver will be examined to establish the locus of the genetic defect. These enzymes also provide a sensitive means of detecting thiazole or pyrimidine synthesis in cell-free systems and will be used to that end.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1958

Part B. Honors, Awards and Publications

Publications other than abstracts from this project:

Eisenberg, F., Jr. and Leder, I. G. An improved scanner for radioactive paper strips. Anal. Chem. (in press).

Honors and Awards relating to this project: None

Serial No. NIAMD- 23
1. Biochemistry & Metabolism
2 Intermediary Metabolism
3. Bacteria

PHS-1111
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Studies on the Enzymatic and Non-Enzymatic Hydrolysis of Conjugated Glucuronic Acids; The Biosynthesis of Inositol.

Principal Investigator: Dr. F. Eisenberg, Jr.

Other Investigator: Dr. Y. Imai

Cooperating Units: None

Man Years (calendar year 1958):
Total: $3\frac{1}{2}$
Professional: 2
Other: $1\frac{1}{2}$

Project Description:

Objectives - To study the action of β-glucuronidase on various types of O-substituted glucuronic acids as an approach to defining the nature of the active site of the enzyme.

To elucidate the mode of biosynthesis of inositol in the mammal.

Methods Employed - Benzoyl glucuronic acid and menthyl glucosiduronic acid were hydrolyzed with purified calf liver β-glucuronidase and non-enzymatically at various pH's in H_2O^{18}. Split products were isolated and converted to CO_2, which was then analyzed for O^{18} in the mass spectrometer.

Glucose variously labeled with C^{14} was administered to rats. Three hours later the animals were sacrificed and inositol was isolated from the whole animal.

Major Findings - In the enzymatic hydrolysis of both substrates in H_2O^{18} only the glucuronic acid was labeled showing that the cleavage occurs at the bond between the anomeric carbon atom and the oxygen. Variation in pH on either side of pH 5, the optimum for the enzyme, had no effect on the mode of cleavage. Hydrolysis of both substrates in N/1 HCl yielded the same results as the enzymatic hydrolysis. In contrast to enzymatic and acid hydrolysis, 0.02 M NaOH and neutral hydroxylamine cleaved benzoyl glucuronic acid on the acyl side of the alkaline hydrolysis.

Although the substrates are structurally dissimilar, menthyl glucosiduronic acid being an acetal and benzoyl glucuronic acid resembling an ester, they are apparently cleaved by the same enzymatic mechanism. Two possible

mechanisms can account for the observed O^{18} labeling in both experiments. An electrophilic attack on the oxygen atom resulting in an intermediate carbonium ion or a nucleophilic attack by the enzyme on the anomeric carbon atom resulting in a net displacement of the aglycone by OH. The former mechanism is observed in acid catalyzed hydrolysis of acetal and can account for the observed results of the acid hydrolysis of the two substrates.

Labeled inositol has been isolated from rats after the administration of glucose 1-C^{14}, 2-C^{14} and 6-C^{14}. Together with the observation that inositol can be cleaved to glucuronic acid in the kidney, these results suggest a pathway of glucuronic acid synthesis from glucose via inositol.

Significance to NIAMD Research - In view of the wide distribution of glucuronic acid in connective tissue, a defect in the metabolism of this compound may be associated with arthritis and other diseases of that tissue. In order to recognize any derangement at the biochemical level in the diseased state it is essential to study the normal course of metabolism.

Proposed Course of Project - If β-glucuronidase is also a transferase with respect to acyl substituted glucuronic acids then the nucleophilic displacement mechanism rather than the carbonium ion mechanism would account for the O^{18} distribution. Experiments will be performed in an attempt to demonstrate this transferase activity.

Chemical and enzymatic degradation procedures for inositol are being tested. When a suitable method is found the labeled inositol will be degraded. From these results it should be possible to deduce a pathway of biosynthesis of inositol.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1958

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Eisenberg, F., Jr. Preparation of the alkaline absorbant for radioactive CO_2 in liquid scintillation counting. Liquid Scintillation Counting, p. 1-3, London, Pergamon Press: 1958.

Eisenberg, F., Jr., Dayton, P. G. and Burns, J. J. Studies on the glucuronic acid pathway of glucose metabolism. J. Biol. Chem. (in press).

Honors and Awards relating to this project: None

Serial No. NIAMD- 24
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Metabolic Fate of Intracellularly Generated Reduced Di- and Tri-phosphopyridine Nucleotides

Principal Investigator: Dr. Ben Bloom

Other Investigator: Dr. J. H. Bryant

Cooperating Units: None

Man Years (calendar year 1958):
Total: 1½
Professional: 1
Other: ½

Project Description:

Objectives - The object of this project is to gain information concerning the metabolic fate of "various" reduced diphosphopyridine nucleotides (DPNH), and "various" reduced triphosphopyridine nucleotides (TPNH) generated intracellularly.

Methods Employed - 1) Those generally used for the specific tritium labeling of substrates or when needed, the development of same. 2) Standard techniques as in current use for in vitro studies involving tissue slices. 3) Analysis of various metabolic products isolated from the in vitro incubations for tritium content.

Major Findings - The findings to date, while of a preliminary nature, have brought to light new information about the existence in cells of discrete functioning DPNH pools and have suggested the undertaking of experiments which will further reveal the intricacies of reductive biosynthetic processes. The results indicate that in the mammalian liver (rat) DPNH generated from glycerol-2-H^3 is more closely coupled to the reductive step in glucose synthesis than is that DPNH formed from lactate-2-tritium. Further, whereas in rat kidney slices the coupling mentioned above can still be detected, there is an additional coupling between fatty acid synthesis and the DPNH formed from lactate-2-tritium.

Significance to NIAMD Research - With the establishment of the behavior of intracellularly DPNH and TPNH in the tissues from normal animals the background will be available for the furtherance of our appreciation of those disease states which comprise the category of metabolic disorders.

Proposed Course of Project - A continuation of the study of the behavior of DPNH generated from glycerol-2-H^3 and lactate-2-H^3 in tissues other than liver and kidney and the utilization of tissues derived from animals characterized by posessing an experimental disease. The synthesis of a select group of tritium labeled compounds which from the vantage point of current knowledge would generate labeled DPNH and TPNH intracellularly.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1958

Part B. Honors, Awards and Publications

Publications other than abstracts from this project:

Bloom, B. and Topper, Y. J. Absolute configuration of enantiomorphic carbanions involved in the aldolase and triose phosphate isomerase reactions. Nature, 181, 1128-1129 (1958).

Honors and Awards relating to this project:

Recipient - Travel award to the International Congress of Biochemistry, Vienna, Austria - September, 1958.

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Metabolism of Tumor Tissues

Principal Investigators: Dr. M. W. Nirenberg and Dr. J. H. Bryant

Other Investigators: Dr. D. Stetten, Jr. and Dr. M. Potter

Cooperating Units: National Cancer Institute (Serial No.)

Man Years (calendar year 1958):
Total: 2-2/3
Professional: 1-2/3
Other: 1

Project Description:

Objectives - To answer the questions (1) do ascites tumors and tumors in tissue culture contain phosphorylase activating enzymes, and (2) will a viral infection causing the production of a tumor have an effect upon the level of phosphorylase?

To determine the biochemical site of action of certain steroids which cause recession of tumor size in a "steroid-sensitive" mouse lymphosarcoma, to compare these effects with those of the same steroids in a "steroid-resistant" variant of the same tumor, and to attempt to ascertain a mechanism for the development of resistance.

Methods Employed - Tumor homogenates will be incubated with dephosphophosphorylase obtained from normal dog liver to determine whether a dephosphophosphorylase kinase is present. The presence of dephosphophosphorylase in tumors can be assayed by incubating tumor homogenates with dog liver dephosphophosphorylase kinase. The presence of the phosphorylase inactivating enzyme in tumors can be determined by incubating phosphorylase with tumor extracts and measuring the rate of inactivation. The Rous chicken sarcoma will be used to study the effects of a viral infection upon the phosphorylase levels of chicken muscle. The activity of the phosphorylase activating enzymes in the Rous sarcoma will also be studied using similar techniques.

Tumor tissue, furnished by Dr. Michael Potter of the NCI (Serial No.), is being studied using *in vivo* and *in vitro* techniques. The metabolism of various isotopically labeled substrates by tumor tissues is determined in the presence and in the absence of certain steroids. Certain metabolic inhibitors, such as CN^-, are used to help localize the steroid effects.

Major Findings - Results to date reveal that the tumors possess the phosphorylase activating enzymes but specifically lack the two forms of phosphorylase, i.e., phosphorylase and dephosphophosphorylase. Experiments with the Rous virus have shown that whereas normal chicken muscle contains high levels of phosphorylase, the Rous sarcoma has negligible activity.

Preliminary results indicate a steroid inhibition of hexose utilization which is active either at a point in the metabolic scheme prior to the formation of glucose-6-phosphate or on both the glycolytic and oxidative pathways of glucose degradation. There is some steroid specificity. These results apply to the "steroid-sensitive" tumor tissue. The "steroid-resistant" tumor has not been available for experiments to date.

Significance to NIAMD Research - All of the ten different types of tumors tested fit into a similar pattern which is distinctly, and apparently qualitatively, different from most normal tissues. The tumors resemble biochemical mutants insofar as all have a similar block in the normal hormonally-linked sequence of phosphorylase activation. These findings therefore point out a difference in an enzymic pathway between most normal and neoplastic cells and, further, help clarify the unknown factors which control the extraordinarily high carbohydrate utilization of tumor cells.

A significant aspect of this project is an attempt to demonstrate a mechanism of hormonal action in tumor tissue. A second aspect of potential importance would be to explain whether the resistance to steroids, which in this case developed from a sensitive tumor which had been treated repeatedly with small doses of steroid, represents a survival of previously existing resistant cells, or a steroid induced change in the genetic character of the sensitive cells.

Proposed Course of Project - Since a specific viral infection has now been found to result in a particular enzymic defect, the way is now open to follow on an enzymatic level the effect of the virus upon the host cell and the concommitant conversion of a normal cell into a tumor cell.

Findings with the steroid-sensitive tissue must be extended and compared with results of experiments to be done with steroid-resistant tissue.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1958

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Nirenberg, M. W. An enzymic defect in ascites tumor cells. Biochim. et Biophys. Acta, 30, 203-204 (1958).

Honors and Awards relating to this project: None

Serial No. NIAMD- 26
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Studies on the Degradation of Insulin and Insulin Derivatives by Mammalian Tissues

Principal Investigators: Dr. F. Tietze and Dr. G. E. Mortimore

Other Investigators: Dr. D. Stetten, Jr., Dr. T. Shiota and Dr. J. E. Folk

Cooperating Units: Clinical Endocrinology Branch, NIAMD (Serial No. [illegible])
Laboratory of Oral and Biological Chemistry, NIDR
(Serial No.)

Man Years (calendar year 1958):
Total: 3
Professional: 2-1/3
Other: 2/3

Project Description:

Objectives - Further studies on the fate of insulin-I^{131} in perfused rat liver have been carried out, with particular emphasis directed to the kinetics of the degradation process and to the nature of radioactive end products.

Previous studies have shown that the reaction of β-bromoethylamine with protein -SH groups leads to the formation of residues of S-(β-aminoethyl) cysteine which are susceptible of cleavage or removal by trypsin or carboxypeptidase-A. These observations have been further extended to the determination, by purely enzymatic means, of the C-terminal tripeptide sequence of the crystalline protein lysozyme.

Methods Employed - Oxygenated whole rat blood, containing added trace quantities of insulin-I^{131}, is cyclically pumped through surviving rat liver. Aliquots of the perfusing medium are withdrawn at intervals of time and assayed for TCA-soluble radioactivity (representing degraded insulin-I^{131}) and TCA-precipitable radioactivity (representing undegraded insulin-I^{131}). Aliquots of the perfusing medium have also been subjected to paper chromatography in an effort to determine the nature of end-products of insulin-I^{131} degradation.

Deleucinated lysozyme, i.e., lysozyme devoid of its C-terminal leucine residue, has been reduced with thioglycolate and subsequently coupled with β-bromoethylamine. The resulting product has then been reacted with carboxypeptidase-B and the liberated amino acids identified by paper chromatography.

Major Findings - A detailed consideration of the kinetic aspects of the degradation of insulin-I^{131} by perfused rat liver has led to the conclusion that approximately 40% of insulin-I^{131} presented to liver is degraded in one passage through this organ. Paper chromatography has revealed that inorganic iodide constitutes a major end product of insulin-I^{131} degradation by liver. That extensive proteolytic degradation of insulin-I^{131} must precede liberation of iodide is, however, shown by the complete suppression of iodide formation when the perfusion medium contains added excess amounts of unlabeled 3-monoiodotyrosine and 3,5-diiodotyrosine.

Reaction of S-(β-aminoethyl) deleucinated lysozyme with carboxypeptidase-B has been found to result in the liberation of arginine and S-(β-aminoethyl) cysteine, whereas deleucinated lysozyme itself yields only arginine. These observations, in conjunction with the known release of leucine by the action of carboxypeptidase-A on the native protein, indicate that lysozyme has the C-terminal tripeptide structure:

.....cystinal·arginyl·leucine.

This conclusion is in agreement with that of other workers employing more conventional techniques.

Significance to NIAMD Research - The rapid and extensive degradation by liver, of insulin-I^{131}, and therefore presumably of the native hormone itself, suggests the probable importance of this organ in the maintenance of hormonal blood level. These results have raised the intriguing possibility of employing, for clinical purposes, insulin derivatives which, while possessing full biological activity, can no longer be attacked by the insulin-inactivating system of liver.

The coupling of -SH groups of proteins with β-bromoethylamine results in the formation of an altered cysteine residue susceptible to cleavage by proteolytic enzymes. This certain proteins, such as insulin, containing in the native state only limited points of attack by trypsin may be altered in such a way as to make available other specific regions of cleavage by this agent. Such an extension of specific degradative regions is of great utility in problems of protein structure.

Proposed Course of Project - An investigation will be made of possible derivatives of insulin, e.g., sulfated insulin, which, although biologically active, may possess an enhanced resistance toward the insulin-degrading system of liver and other organs.

Further studies on the specificity of the reaction of β-bromoethylamine with proteins will be carried out.

Part B included: Yes

Individual Project Report
Calendar Year 1958

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Shiota, T., Folk, J. E. and Tietze, F. Inhibition of lysine utilization in bacteria by S-(β-aminoethyl) cysteine and its reversal by lysine peptides. Arch. Biochem. Biophys. 77, 372-377 (1958).

Mortimore, G. E., Tietze, F. and Stetten, D., Jr. Studies on the metabolism of insulin-I^{131} in isolated, perfused rat liver and hind-limb preparations. Diabetes (in press).

Honors and awards relating to this project: None

3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Studies on Oligosaccharides and Polysaccharides

Principal Investigator: Dr. M. R. Stetten

Other Investigators: Dr. D. Stetten, Jr. and Mr. H. M. Katzen

Cooperating Units: None

Man Years (calendar year 1958):

Total: 3
Professional: 2½
Other: ½

Project Description:

Objectives - To gain insight into the normal structure, synthesis and metabolism of certain polysaccharides and oligosaccharides.

Methods Employed - Maltose-C^{14} and maltotriose-C^{14} are made from glucose-C^{14} by transglucosylation reactions using enzyme preparations from rat liver. These compounds are isolated chromatographically, characterized by preparation of derivatives, and the position of the isotopic label within the oligosaccharides located by the preparation of flavonole derivatives or of oxidative and hydrolytic products. The properties and substrate specificity of the enzyme are studied.

Glycogen is isolated from animals by acidic methods, purified and used in studies of the nature of the changes which occur on treatment with alkali under various conditions. Light scattering methods are used in following the decline in molecular weight and chemical and chromatographic methods are used for identification of the products produced.

Major Findings - A rat liver enzyme, called transglucosylase, has been found to be capable of transferring glucosyl residues from maltose, maltotriose or glycogen to glucose, maltose and higher homologues, or to water. Glucose-C^{14} is introduced by the enzyme into only the reducing terminal sugar of the oligosaccharides. The glucosyl transfer from one sugar to another is reversible but that to water is irreversible. Phosphate and other dialyzable cofactors do not participate.

Significance to NIAMD Research - Alterations and defects in the way the body metabolizes various carbohydrates have been found to be characteristic of certain nutritional states, drug actions and metabolic diseases.

Any additional knowledge as to how carbohydrates are normally handled may be expected to contribute to a better understanding of the nature of these conditions and diseases.

Proposed Course of Project - The purification and properties of the mammalian transglucosylation enzyme will be further studied.

A stumbling block in the study of the degradation of polysaccharides by alkalies has been the introduction of extraneous materials produced by the prolonged action of alkali on glass and other vessels used. This has now been eliminated by the use of specially made teflon vessels. The nature and the rate of the degradation of glycogen by alkali under different conditions will be studied and attempts made to characterize the saccharinic acids produced.

Part B Instructions: None

PHS-NIH
Individual Project Report
Calendar Year 1958

Part B. Honors, Awards and Publications

Publications other than abstracts from this project:

Stetten, M. R., Katzen, H. M. and Stetten, D., Jr. A comparison of the glycogens isolated by acid and alkaline procedures. J. Biol. Chem. 232, 475-488 (1958).

Stetten, M. R. and Stetten, D., Jr. Influence of molecular size of glycogen on the phosphorylase reaction. J. Biol. Chem. 232, 489-504 (1958).

Stetten, D., Jr. and Stetten, M. R. Homopolysaccharides, in "Polysaccharides in Biology", Josiah Macy, Jr. Foundation, New York, N Y., p. 9-153 (1958).

Stetten, M. R. Transglucosylation by a mammalian liver enzyme. J. Am. Chem. Soc. (in press).

Honors and Awards relating to this project: None.

PHS-NIH
Individual Project Report
Calendar Year 1958

PART A.

Project Title:

A. Studies on Steroid Reduction

B. Mechanism of Steroid Hydroxylation

C. Mechanism of Action of Steroid Hormones

D. Studies on oxidation of cyclic secondary alcohols

Principal Investigator: Gordon M. Tomkins

Other Investigators: Joseph S. McGuire
K. L. Yielding
Vincent Hollis
A. D. Merritt
Jean Curran

Cooperating Units:

Man Years (Calendar year 1958):
Total 4
Professional: 3
Other 1

Project Description:

Project A

OBJECTIVES - The saturation of the 4,5 double bond of ring A of the steroid nucleus is the rate-determining step in the biological inactivation of many potent steroid hormones. In the course of this reduction, an asymmetric center at C5 is produced and both isomers (5α and 5β) have been detected as metabolic products. Previously, we have described a series of enzymes in liver cytoplasm which will affect this reduction - each one being highly substrate specific - to produce the 5β isomer. More recently, preliminary studies in another institution have indicated the existence of a microsomal enzyme in liver which produces the 5α isomer. We have undertaken a study of the questions:

Part B included Yes

1. What factors govern the rate and steric course of steroid reduction?

2. What is the biological (i.e., functional and genetic) relationship between the 5α- and 5β- steroid reducing enzymes?

Finally, since these compounds are highly active biologically, we have undertaken a study of the possible consequences of steroid reduction for other reactions that could be coupled to it.

METHODS AND MAJOR FINDING - Rats were treated with thyroxin and it was found that steroid reduction by liver homogenates was increased following 3 days of such treatment. TPNH is necessary for this reduction and was found to be rate-limiting in homogenates. The increased rate of reduction was caused by increased available TPNH due to the induction of G-6-P dehydrogenase in liver by thyroxin.

Following larger periods of thyroxin treatments however, a several fold increase in a microsomal - Δ^4-3-keto steroid dehydrogenase (5α) was found to occur in liver. There was no alteration in the activity of a soluble Δ^4-3 keto steroid hydrogenase (5β) also present exclusively in liver.

On the basis of varying ratios of activity with different steroid substrates from preparation to preparation and with different treatment, it appears that there are several microsomal steroid hydrogenases (5α). This is the situation with the soluble (5β) hydrogenase. TPNH has been found to be a necessary and exclusive cofactor for the particulate enzyme. Data for cofactor requirements for the soluble enzyme is not conclusive. Efforts to carry out the reverse (dehydrogenating) reaction with both enzymes has not been successful. In a rat hepatoma that will reduce only one of the substrates tested, the activity appears in both the soluble and particulate fraction. The above suggest a close relationship between the particulate and soluble enzymes, and efforts to solubilize the particulate enzyme as well as to adsorb the soluble enzyme onto particles and so effect a reversal of the isomeric configuration of their reaction products are being made.

Using C_1 and uniformly labeled glucose C^{14} as substrate, rat liver slices and crude homogenates showed significant stimulation of $C^{14}O_2$ production when incubated in the presence of various steroids in concentrates as low as 5×10^{-6} M. Addition of large amounts of

hexokinase served only to enhance the effects, and similar stimulation was observed with G-6-P as substrate. Furthermore direct assays of hexokinase failed to reveal any steroid stimulation. Such experiments were done using conditions under which glucose was presumed to be oxidized almost exclusively via the TPN specific hexosemonophosphate shunt.

Finally, working with ultracentrifugate of rat liver it was possible to show that stimulation of CO_2 production from glucose-6-phosphate resulted from steroid ketone ring reduction by the mechanism of TPN production. (Steroid reduction is TPNH specific) Steps in proof included: (1) abolishing the effect with tremendous excesses of TPN, (2) correlation of individual steroid reductases with the degree of stimulatory effect shown by the steroid; (3) stoichiometry of the overall reactions. Furthermore, the hexose-monophosphate shunt was not stimulated in tissue lacking steroid reductases.

Project B.

OBJECTIVES - Previous investigations have shown that the introduction of molecular oxygen into the steroid nucleus is a complicated enzymatic reaction. It is of interest from the standpoint of mammalian physiology since hormone and sterol biosynthesis requires many similar steps. It is also of probable importance in microbial physiology since a great variety of molds, yeasts, and a few bacteria can catalyze this reaction as well. A study of its mechanism, undertaken several years ago, has continued.

METHODS AND MAJOR FINDINGS - Further characterization of the enzymes from mammalian adrenal has been difficult because of their lability and primarily the action inhibitors, etc. has been studied. Because of the difficulty of this approach, a more intensive microbial study has been undertaken.

In this recently started project a strain of Rhizopus nigricans has been found to convert 11-desoxycorticosterone into predominantly a compound tentatively identified as the 11α-hydroxy form (Eq. 1) of desoxycorticosterone (epi-cortisterone) This conversion is obtainable in a semi-synthetic media. Washed mycelia can be obtained which show readily assayable activity in crude minces. Using this as an example of 11-hydroxylation, attempts are being made to isolate the activity in cell-free extracts, purify the enzyme(s) and study the mechanism of hydroxylation.

Project C.

OBJECTIVES - The steroid hormones have pronounced effects on growth, differentiation and other essential biological processes. Little is known about the molecular mechanisms by which they exert their dramatic effect and the present studies were undertaken to study this problem.

METHODS AND MAJOR FINDINGS - Lactate and pyruvate oxidation - $C^{14}O_2$ production from C_2 labeled (and to a lesser extent C_1 labeled) pyruvate and lactate by rat liver homogenate was significantly enhanced by cortisone and testosterone in concentrations as low at 10^{-7} M. Addition of TPN was essential for such an effect and ATP in high concentration would abolish the effect. Interestingly, liver homogenates from fasted animals either showed no effect by steroid or showed inhibition. Preliminary screening with a large group of steroids does not show good correlation with steroid reduction activity, or the potential action of the steroid as a transhydrogenase cofactor. Further work is actively in progress.

Project D.

OBJECTIVES - Cyclic secondary alcohols are extremely useful model compounds with which to study steroid metabolism, since kinetic and equilibrium factors can be controlled by appropriate substitution. In addition, crystalline horse liver alcohol dehydrogenase has been extensively studied from an enzyme standpoint. The present study represents a fusion of these two interesting areas.

METHODS AND MAJOR FINDINGS - Previous investigations indicated that cyclic secondary alcohols were able to oxidize reduced diphosphopyridine nucleotide in the presence of a rat liver homogenate and studies were undertaken to purify the enzyme responsible for this activity. A partially purified enzyme was prepared and its kinetics, pH optimum, equilibrium constants, and substrate specificity were studied. Cyclohexanone was found to be stoichiometrically and reversibly reduced by diphosphopyridine nucleotide. The products of the reaction were identified by means of paper chromatography. Since the substrate specificity paralleled that of horse liver alcohol dehydrogenase, the enzyme was thought to be a liver alcohol dehydrogenase. Accordingly, the crystalline horse liver enzyme was investigated and it was found that cyclohexanol was a somewhat better substrate than ethanol on a molar basis. The kinetics of this reaction were examined.

Methyl substituted cyclic alcohols and ketones also were found to be active with liver alcohol dehydrogenase. The additional substituents allow the formation of cis- and trans- isomers which may be identified by means of gas chromatography and infrared spectroscopy. The conformation of cyclic ketone reduction products of this enzymic activity will be studied to further define enzyme-substrate interactions.

Part B:

Publications other than abstracts from this project:

Axelrod, J., Inscoe, J. and Tomkins, G. Enzymatic Synthesis of N-glucuronic Acid Conjugates. J. Biol. Chem., 232, 835 (1958).

Tomkins, G., Curran, J. and Michael, P. Further Studies on Enzymatic Adrenal 11-β Hydroxylation. Biochim. Biophys. Acta, 28, 449 (1958).

McGuire, J., and Tomkins, G. Effect of thyroxin administration on the rate and steric course of enzymatic reduction of steroids. Nature 182, 261 (1958).

Tomkins, G. Studies on the mechanism of steroid hydroxylation. Colloquium on Oxygenating Enzymes, 4th Int. Congress of Biochemistry, Vienna, Sept. 1958. Pergammon Press, London (In press).

McGuire, J. S., Tomkins, G. M., The Effects of Thyroxin Administration on the Enzymic Reduction of Δ^4-3-ketosteroids. J. Biol. Chem., (In press).

Serial No. NIAMD 29
1. Biochemistry & Metabolism
2. Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

PART A.

Project Title:

Guanosine diphosphate fucose from Aerobacter aerogenes

Principal Investigator: Victor Ginsburg
(Departed NIH, Sept. 1958)

Other Investigators: H. N. Kirkman (first part)

Cooperating Units:

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

OBJECTIVES - To see if a fucose-containing nucleotide could be found in a bacterial strain elaborating a fucose-containing polysaccharide and to see in what enzymatic steps the nucleotide was involved.

METHODS AND MAJOR FINDINGS - Since nucleotides are already known to be involved in the synthesis of some di- and polysaccharides, as well as the interconversion of some monosaccharides, it was believed that some nucleotide of fucose might be present in cells producing a fucose-containing polysaccharide. Such a nucleotide was unknown at the start of the project. Choosing Aerobacter aerogenes, A_3S_1 (ATCC 12657) as a possible source, the investigators isolated the nucleotides from 150 g. of the freshly-grown bacteria by ethanol extraction, mercuric precipitation, and chromatography on a Dowex 1-Cl^- column. Paper electrophoresis and paper chromatography of the peak corresponding to uridine diphosphate glucose and uridine diphosphate galactose revealed a third nucleotide showing the ultraviolet absorption spectrum of a guanosine derivative and the Dische color test of fucose. Hydrolysis of this nucleotide revealed paper chromatographically identifiable fucose and guanosine diphosphate.

Part B included Yes

The second part of the study led to the discovery of the conversion of guanosine diphosphate mannose, the only other known guanosine nucleotide, to guanosine diphosphate fucose by crude lysates of the Aerobacter aerogenes in the presence of TPNH. Further studies of this phenomenon are planned by the principal investigator.

SUMMARY AND SIGNIFICANCE TO THE INSTITUTE - The discovery of the new nucleotide, guanosine diphosphate fucose, and its conversion from guanosine diphosphate mannose, increases and almost completes the variety of nucleotides carrying a monosaccharide which occurs simultaneously in the polysaccharides of cells. These findings may provide a stimulus for new ideas concerning the mechanism of synthesis of complex polysaccharides and will provide an additional tool for such studies. Fucose is a component of several polysaccharides of clinical and immunological importance.

PART B:

Publications other than abstracts from this project:

Ginsburg, V. and Kirkman, H. N., Isolation of Guanosine Diphosphate Fucose from Aerobacter aerogenes, J. Am. Chem. Soc., 80, 3481 (1958).

Ginsburg, V., Formation of Guanosine Diphosphate Fucose from Guanosine Diphosphate Mannose, J. Am. Chem. Soc., 80, 4426 (1958).

Serial No. NIAMD 30
1. Biochemistry & Metabolism
2. Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title:

Protein Synthesis

Principal Investigator: Hubert de Robichon Szulmajster

Other Investigators:

Cooperating Units: Carnegie Institute - Department of Terrestrial Magnetism

Man Years (Calendar year 1958):
Total 1/2
Professional 1/2
Other

Project Description:

OBJECTIVE - Protein synthesis is the most characteristic and important biosynthetic reaction. Microorganisms, past culicly amino acid requiring mutants, offer an ideal system for the study of this process.

METHOD AND MAJOR FINDINGS - A methionine-requiring E. coli mutant was used. If up to 70% of the methione is replaced by an analog, norleucine, growth continues at a linear rather than at an exponential rate. Yet, an adaptive enzyme, β-galactosidase can be synthesized in normal amounts. Also, glucose-6-phosphate dehydrogenase is synthesized under these conditions. The replacement of methione by norleucine seems to be universal, i.e., all protein fractions obtainable after chromatography on a DEA column contain about the same ratio of norleucine to methione. The findings were similar when either norleucine or p-fluorophenylalanine were used with wild type E. coli.

Part B included No

Serial No. NIAMD 31
1. Biochemistry & Metabolism
2. Metabolic Enzymes
3. Bethesda

HHS-NIH
Individual Project Report
Calendar Year 1958

PART A.

Project Title:

A. Enzymatic studies of the heterozygous state in galactosemia.

B. Isolation and comparative studies of the Glucose-6-phosphate dehydrogenase from normal and primaquine-sensitive erythrocytes.

C. Modification of the enzymatic diagnostic test for galactosemia to estimate galactose-1-phosphate accumulation in erythrocytes.

Principal Investigator: Henry J. Kirkman
A. Herman M. Kalckar, in part

Cooperating Units:

Man Years (Calendar year 1958):

Total:	1-1/2
Professional:	1
Other:	1/2

Project Description:

Project A.

OBJECTIVES - To determine if the heterozygous state manifests itself in galactosemia by a discernible diminution in the activity of erythrocytic galactose-1-phosphate uridyl transferase, the enzyme known to be absent or defective in congenital galactosemia.

METHODS AND MAJOR EQUIPMENT - In order to measure quantitatively the amount of galactose-1-phosphate uridyl transferase (Eq. 1) in erythrocytes it was necessary to devise (1957 project) a new assay which permitted continuous following of the reaction

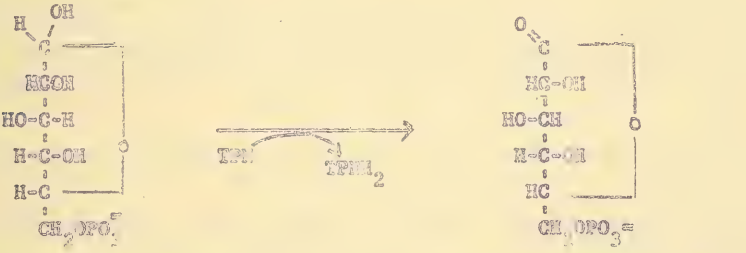
Eq. 1. Galactose-1-phosphate + uridine diphosphate glucose $\rightleftharpoons$ uridine diphosphate galactose + glucose-1-phosphate

under conditions yielding zero-order kinetics, with less than 5-10% error. When methylene blue and various adjuvants are added to hemolysates, they will consume oxygen in amounts proportional to the amount of glucose-1-phosphate generated and at a rate which makes Eq. 1 rate-limiting. The assay, based upon this effect, revealed that the parents of galactosemic infants have lower activities of the galactose-1-phosphate uridyl transferase in erythrocytes than do normal control. Thirteen of the 18 parents fell below the + 2 standard deviation range of the normals. Other members of one family were also tested and found to show intermediate activities in a pattern compatible with a simple recessive inheritance.

Project B.

OBJECTIVES - To devise a method for purifying glucose-6-phosphate dehydrogenase from human erythrocytes, to study the characteristics of this enzyme, and to see if it differs qualitatively from the enzyme from primaquine-sensitive erythrocytes, known to have only 10-20% of the normal activity in glucose-6-phosphate dehydrogenase Eq. (2).

Eq. 2



D Glucose-6-phosphate

6-phosphogluconolactone

METHODS AND MAJOR FINDINGS - By means of differential absorption on an anion-exchange resin, elution, and ammonium sulfate fractional precipitation, it has been possible to isolate from human erythrocytes this enzyme in a hemoglobin-free form with 80-fold purification to date. The enzyme, like that from yeast, is very labile. Means of protecting and storing the enzyme have been found. Its enzymatic properties - K_m's, pH-optimum curve, lability under various conditions, inhibitors, movement on ion-exchange chromatography and electrophoresis - have been or are being studied. In all respects so far studied, the normal and primaquine-sensitive enzyme seem alike. No evidence is therefore present that the lower activity of the reaction (Eq. 2) in primaquine-sensitive erythrocytes is due to "wasting" or qualitative alterations of the enzyme. Carson and Alving have found that erythrocytic stroma will

inactivate the glucose-6-phosphate dehydrogenase in crude hemolysate from primaquine sensitive individuals but not in crude hemolysates from normal individuals. When the enzyme purified by the above method is added to stroma, in the presence of primaquine-sensitive hemolysate, the enzyme is inactivated regardless of whether it is purified from normal or primaquine-sensitive blood. The investigator is in communication with Dr. Carson, and a brief, further exploration of the last effect is planned, perhaps jointly.

Project C.

OBJECTIVES - To devise a simple method for estimating the amount of galactose-1-phosphate accumulated in the erythrocytes of galactosemic children.

METHODS AND MAJOR FINDINGS - Although Holzel, Komrower, et al. have devised a paper chromatographic method for estimating the amount of galactose-1-phosphate in erythrocytes, a preliminary study reveals that the enzymatic, diagnostic test for galactosemia devised in this institution by Kalckar and co-workers can be slightly modified to provide an alternate method for estimating the amount of galactose-1-phosphate present in erythrocytes of galactosemic individuals. Only small amounts of blood (2 ml.) are required.

Other Projects, Collaborative - Isolation of guanosine diphosphate fucose. See Individual Project Report for Victor Ginsburg.

SUMMARY AND SIGNIFICANCE TO THE FUTURE - The detection of intermediate levels of galactose-1-phosphate uridyl transferase in parents and relatives of galactosemic children constitutes the first evidence, at an enzymatic level, of the existence of a "trait", or heterozygous state, in galactosemia. On the basis of these findings, it has been proposed by Dr. Kalckar that congenital galactosemia be renamed "hereditary galactosemia". The new assay makes possible the study of family pedigrees and mode of inheritance of the disease.

In primaquine-sensitivity one encounters an inherited metabolic defect of erythrocytes which is characterized, among other things, by a deficiency, but not a total absence, of activity of glucose-6-phosphate dehydrogenase. Whether the affected erythrocyte is endowed with less enzyme, a "warped" and inefficient enzyme, or an intracellular inactivator of the enzyme seems discernible by the methods mentioned and may expand current understanding of the genetic influences on enzymes.

Periodic estimation of the amount of galactose-1-phosphate present in the erythrocytes of galactosemic individuals is of considerable clinical importance. It is presently believed that the galactosemic individual can lead a normal life if he is deprived of galactose. Exposure to galactose results in cataracts, growth failure, irreversible degrees of brain-damage and, often, death. Because many modern foods often contain unmentioned milk with its galactose-containing sugar, and, because of the clandestine eating habits of some children, a tight enforcement of a galactose-free diet is difficult. This alternate method of measuring galactose-1-phosphate in erythrocytes provides a direct measure of the substance, the galactose-1-phosphate itself, believed by many to be a major cause of the cellular injury in galactosemia. This substance persists for several weeks after the individual consumes galactose. The test would be particularly convenient for clinical centers in this country, where the reagents for the diagnostic test are already commercially available and are likely to be used in the initial diagnosis

PART B

Publications other than abstracts from these projects:

Kirkman, H. N. and Kalckar, H. M., Enzymatic deficiency in congenital galactosemia and its heterozygous carriers. Annals N. Y. Acad. Sciences, 75, 274 (1953).

Kirkman, H. N. and Bynum, E., Enzymatic evidence of a galactosemic trait in parents of galactosemic children. Annals of Human Genetics of Great Brit., Feb. 1959, in press.

Serial No. NIAMD 32
1. Biochemistry & Metabolism
2. Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

PART A.

Project Title:

A. Galactose-Glucose Interconversion

B. Role of UDPGal as a Galactosyl Donor

Principal Investigator: Elizabeth S. Maxwell

Other Investigators: Herman M. Kalckar
Huguette de Robichon-Szulmajster

Cooperating Units:

Man Years (Calendar year 1958):
Total 2
Professional: 1-1/2
Other 1/2

Project Description:

A. Galactose-Glucose Interconversion

OBJECTIVES - Previous studies in this laboratory demonstrated that UDPGal-4-epimerase purified from calf liver has an absolute requirement for DPN and is inhibited by DPNH. This requirement for DPN suggested that the inversion of configuration at carbon-4 proceeds by oxidation-reduction. Attempts to show unequivocally that such a mechanism occurs were unsuccessful. It was not possible to demonstrate DPNH formation under a wide variety of conditions. No enzymic incorporation of tritium into the uridine nucleotide occurred when the reaction was run in the presence of DPN or DPNH labeled in the para position.

The present studies using UDPGal-4-epimerase from yeast were undertaken to investigate further the mechanism of the inversion catalyzed by this enzyme. These studies were carried out in collaboration with Dr. H. M. Kalckar and Dr. Huguette Szulmajster.

Part B included Yes

METHODS AND MAJOR FINDINGS - A procedure for the purification of UDPGal-4-epimerase from galactose-adapted S. fragilis was developed. About 100-200 fold purification was obtained by ammonium sulfate fractionation followed by chromatography on DEAE cellulose. The activity of the most purified fraction was not stimulated by the addition of DPN nor was the enzyme inhibited by DPNH. It was found that yeast grown on galactose contained a fluorescent protein which accompanied the epimerase on purification. The ratio of fluorescence to epimerase activity remained nearly constant during the purification. Yeast grown on glucose contained only traces of UDPGal-4-epimerase activity and very little of the fluorescent material. The fluorescence exhibited was similar to that of free DPNH with an activation maximum at 350 mμ and an emission maximum about 450 mμ. The addition of PCMB to the purified enzyme brought about a disappearance of the fluorescence. When PCMB-treated enzyme was precipitated with ammonium sulfate and freed of PCMB, the addition of DPN was now necessary for UDPGal-4-epimerase activity. Treatment of the purified enzyme with darco removed only a fraction of the fluorescence and DPN was not required after such treatment. However, elution of the darco with 50% ethanol yielded a product, presumably DPN, which was able to activate purified calf liver 4-epimerase. The data suggest that yeast UDPGal-4-epimerase contains bound DPN and that the coenzyme may be bound in such a way that it exhibits fluorescence similar to that of DPNH.

B. UDPGal as a Galactosyl Donor

OBJECTIVES - This project was initiated to investigate the possibility that UDPGal functions as a galactosyl donor in the biosynthesis of mammalian polysaccharides such as the blood group substances and the lung galactans.

METHODS AND MAJOR FINDINGS - UDPGal labeled in the hexose moiety with C^{14} was used. Two tissues were investigated. Gastric mucosa was chosen because *in vivo* large amounts of galactose-containing blood group substances are secreted by this tissue. It was found that microsomes from guinea pig gastric mucosa incorporated radioactivity from UDPGal into an alcohol precipitable product. Because of the low activity of this system, a similar system from lung was used. Lung is known to contain a polysaccharide consisting solely of galactose. Microsomes from guinea pig lung did actively incorporate galactose into an alcohol precipitate material. However, further purification of the material has shown that it is not a polysaccharide. The nature of the reaction observed and the product or products formed is not known.

Chromatography of the nucleotides from rabbit gastric mucosa failed to reveal any previously undescribed intermediates in polysaccharide synthesis.

SUMMARY -

A. Yeast UDPGal-4-epimerase has been purified and found to be associated with a blue fluorescent material. The purified enzyme does not require DPN. Treatment with PCMB brings about a disappearance of fluorescence as well as activity. The latter can be restored by adding DPN.

B. The role of UDPGal in polysaccharide synthesis in mammalian tissue has been investigated further. Results have not been sufficiently encouraging to continue the project at the present time.

Part B:

Publications other than abstracts from this project:

Maxwell, Elizabeth S., "The Enzymic Interconversion of Uridine-diphosphoglucose and Uridine diphosphogalactose", J. Biol. Chem., 229, 139 (1957).

Maxwell, Elizabeth S., Szumajster, Paquette de Robichon, and Kalckar, Herman M., "Yeast Uridine Diphosphogalactose-4-Epimerase, Correlation between Activity and Fluorescence", Arch. Biochem. and Biophysics (In press).

Kalckar, Herman M. and Maxwell, Elizabeth S., "Biosynthesis and Metabolic Function of Uridine Diphosphoglucose in Mammalian Organism and its Relevance to Certain Inborn Errors" Physiological Reviews 38, 77 (1958).

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

ANNUAL PROJECT REPORT

CALENDAR YEAR 1958

Summary Sheet

LABORATORY OF CHEMISTRY

Estimated Obligations for FY 1959

Total - $1,047,836
Direct - $637,000
Reimbursement - $410,836

Project Numbers 33 thru 67 included

Serial No. NIAMD- 33

1. Chemistry
2. Carbohydrates
3. Bethesda

NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Studies on the Synthesis of Carbohydrate Derivatives for Biochemical and Medical Research.

Principal Investigator: Hewitt G. Fletcher, Jr.

Other Investigators: H. J. Diesl, D. L. MacDonald (V.S. from 8/15/[illegible]), R. M. Wiss, C. Pedersen (Fellow from 5/1/58), E. Vis (Fellow until 1/15/58), H. B. Wood, Jr.

Cooperating Units: None

Man Years (calendar year 1958):
Total: 6 2/3
Professional: 5
Other 1 2/3

Project Description:

General Objectives:

A. To investigate new synthetic pathways for the synthesis of carbohydrate derivatives of biochemical and medical importance.

B. To provide cooperating biochemists and clinicians with otherwise inaccessible carbohydrate substances.

C. To extend knowledge of the chemical properties of biochemically important carbohydrates.

Specific Objectives:

A. To study the chemistry of D-ribose in order to evolve new or improved synthetic pathways to the ribofuranosides and particularly selectively substituted derivatives thereof.

B. To explore the chemistry of 2-deoxy-D-ribose, specifically methods of synthesizing 2-deoxy-D-ribofuranosides and their phosphates of 2-deoxy-D-ribose which have been demonstrated to be (or suspected of being) metabolic intermediates.

C. To synthesize intermediates of the metabolism of carbohydrates other than 2-deoxy-D-ribose.

D. To devise syntheses of 1-O-acyl aldoses.

Progress during 1958:

A. An improved and simplified preparation of 2-deoxy-D-ribose has been devised making this hitherto relatively inaccessible sugar readily available.

B. The physical properties of highly purified 2-deoxy-D-ribose have been examined intensively.

C. 2-Deoxy-D-ribose 5-phosphate, an important metabolic intermediate has been synthesized for the first time (by chemical means).

D. 2-O-Methylsulfonyl-D-arabinose has been synthesized by several routes and obtained for the first time in crystalline form. The presence of a strongly electronegative group at carbon two of this aldose markedly influences the properties of the sugar. The substance, for instance, shows a strong tendency to assume the furanose form, suggesting its potential value for the synthesis of "spurious" nucleosides of possible anti-cancer interest.

E. 2,3,5-Tri-O-benzoyl-D-ribofuranosyl fluoride, the first crystalline fully acylated ribofuranosyl halide has been made.

F. Phenyl β-D-ribofuranoside has been converted to 1,5-anhydro-β-D-ribo-hexulofuranose through the action of alkali. This is the first time that such a ring structure has been synthesized through this type of reaction.

G. Fusion of 1,2,3,5-tri-O-benzoyl-2-O-p-nitrobenzenesulfonyl-β-D-ribofuranose with benzoic acid and sodium benzoate caused (1) loss of the sulfonyl group at carbon two with inversion of configuration (2) migration of the acyl group at carbon one to carbon two and (3) addition of a benzoyloxy group at C_1. Several modifications of this reaction, the first of its type to be carried out in the sugar series, were studied.

Significance of the project to the program of the Institute:

The synthetic methods and the materials produced in the course of this project have been and will be of utility to various research groups in NIAMD.

Proposed course of project:

All items listed under "Specific Objectives" will be pursued during 1959.

Part B included: Yes

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Ness, R. K. and Fletcher, H. G., Jr.: The Anomeric 2,3,5-Tri-O-benzoyl-D-arabinosyl Bromides and Other D-Arabinofuranose Derivatives. J. Am. Chem. Soc. 80: 2007-2010, 1958.

Diehl, H. W. and Fletcher, H. G., Jr.: A Simplified Preparation of 2-Deoxy-D-Ribose. Chem. & Ind. 1958: 1087-1088.

Vis, E. and Fletcher, H. G., Jr.: 1,5-Anhydro-β-D-ribofuranose from Phenyl β-D-Ribofuranoside. J. Org. Chem. 23: 1395, 1958.

Wood, H. B., Jr. and Fletcher, H. G., Jr.: The Preparation of Crystalline 2-O-Methylsulfonyl-D-arabinose and Some of Its Derivatives. J. Am. Chem. Soc. 80: 5242-5246, 1958.

Fletcher, H. G., Jr.: Some Aspects of the Chemistry of Ribose. Record Chem. Progr. 19: No. 3, 147-164, 1958.

Diehl, H. W. and Fletcher, H. G., Jr.: 2-Deoxy-D-ribose. I. A Simplified Preparation of 2-Deoxy-D-ribose Based on Treatment of α-D-Glucose Monohydrate with Solid Calcium Hydroxide. Arch. Biochem. Biophys. 78: No. 2, 0000, 1958.

Serial No. NIAMD- 34

1. Chemistry
2. Carbohydrates
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Higher-Carbon Sugars, Anhydro Sugars, Sugar Alcohols and Their Derivatives.

Principal Investigator: Nelson K. Richtmyer

Other Investigators: Alexander J. Charlson (U.S. from 4/1/58), James W. Pratt, Emmanuel Zissis, Edward W. Tracy, John T. Sipes

Cooperating Units: None

Man Years:
Total: 6 1/3
Professional: 3 2/3
Other: 2 2/3

Project Description:

Objectives: To evolve generalizations relating the physical and chemical properties of the groups of substances named in the project title to their configurations and conformations.

Methods Employed and Major Findings: In continuation of our studies on the formation of monomeric nonreducing anhydro sugars by the action of acids on monosaccharides, we found that L-galactose was converted by 0.2 N aqueous sulfuric acid to 1,6-anhydro-β-D-galactofuranose (0.95%) and 1,6-anhydro-β-D-galactopyranose (0.71%); a smaller amount of a third anhydride, possibly 1,5-anhydro-α-D-galactofuranose, was isolated as a sirup and awaits further investigation (as do the two crystalline anhydro-L-galacto-heptuloses mentioned in the 1957 report). Work on the preparation of 3-deoxy-D-xylo-hexose, 3-deoxy-L-lyxo-hexose, and their anhydrides was continued, as was the transformation of a 1,6-anhydro-β-mannose derivative to the corresponding 1,6-anhydro-D-talose derivative; also, studies on the alkaline degradation of phenyl α-D-manno-heptulopyranoside and of the new phenyl α-D-altro-heptulopyranoside were pursued.

Two heptuloses are known to occur in nature: D-manno-heptulose in the avocado and sedoheptulose (= D-altro-heptulose) in Sedum species (principally, although very small amounts have been reported

from many other plants). In examining aqueous extracts of the Californian avocado (Calavo, Fuerte variety), we have recently isolated the first naturally occurring octulose, have characterized the sirupy sugar as a crystalline 2,5-dichlorophenylhydrazone, and have proved that the lowest three asymmetric carbon atoms have the D-*gluco* configuration. The same octulose (as indicated by paper chromatography) occurs in *Sedum* species and we are currently working (presently half a ton of *Sedum spectabile* in order to obtain more of the new sugar. In addition, both the avocado and *Sedum* species appear to contain still another higher-carbon ketose, and the avocado also appears to contain two new higher-carbon sugar alcohols.

Proposed Course of Project: Continuation of these and closely related topics.

Part B included: Yes

Part B. Honors, Awards, and Publications

Publications other than Abstracts from this project:

Richtmyer, N. K.: The Formation of 1,6-Anhydro-α-D-galactofuranose and 1,6-Anhydro-β-D-galactopyranose by the Action of Acid on D-Galactose. Arch. Biochem. Biophys. 78: No. 2, 0000, 1958.

Serial No. NIAMD 35
1. Chemistry
2. Analgesics
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project title: (a) Transformation of the opium alkaloid thebaine into into medicinally useful drugs (i.e.: analgesic and muscle relaxants).

(b) Elucidation of the structure of "14-hydroxycodeine".

Principle Investigator: Lewis J. Sargent

Other Investigators: None

Cooperating Unit: U. Weiss, NIAMD. Serial No. 106 .

Man years: Total : 1
Professional: 1
Other : 0

Project description:

Objectives: (a) With the view to determining the effect on analgesic activity of altering the point of attachment of the nitrogen ring from carbon-9 to carbon-7 in the morphine system, dihydrocodeinone (derived from thebaine) has been transformed (six steps) to a hitherto unknown class of morphine derivatives in which the nitrogen terminus of the ethanamine chain is presumed to be linked to carbon-7.

(b) The substance "14-hydroxycodeine" has been known for many years but its structure has never been established. In an attempt to clarify this situation, evidence was recently obtained both independently and in collaboration with Dr. Ulrich Weiss of this Institute, that 14-hydroxycodeine is bimolecular.

Methods employed: (a) Dihydrocodeinone methiodide was degraded to the methine which was catalytically reduced (9,10-position) to dihydrocodeinone-dihydromethine. Bromination of the latter in glacial acetic acid afforded the 1,7-dibromodihydromethine hydrobromide. Since the 7-bromine atom is part of an active α-bromoketonic system, treatment of the hydrobromide with aqueous ammonia caused spontaneous cyclisation, the net result presumably being the formation of a cyclic methobromide in which the nitrogen atom is linked to carbon-7. The 1-bromine atom was easily hydrogenolysed; the 6-ketone function was unaffected during this treatment. The carbonyl function could, however, readily be reduced to the carbinol either with sodium borohydride or platinum oxide and hydrogen. The latter is preferred because of its greater stereospecificity. Attempts to arrive at the corresponding tertiary amine (whether 6-carbonyl or 6-carbinol) through dry distillation of the quaternary methobromide resulted in cleavage of methyl bromide along

PHS-NIH
Individual Project Report
Calendar Year 1958

with simultaneous fission of the 4,5-oxygen bridge in both cases. This afforded new analogs of dihydrothebainone and of dihydrothebainol respectively.

(b) In regard to "14-hydroxycodeinone", a Signer molecular weight determination gave a value of 627 (bimolecular). Infrared examination demonstrated the presence of a carbonyl function, while a Zerewitinoff analysis accounted for three active hydrogens (three hydroxyl groups). Only a dimeric structure could accomodate these data.

Major findings: A consequence of the above research has been the construction of an entirely novel ring system within the morphine framework. This has opened up new research areas which may lead to clinically useful drugs. One of the intermediate derivatives (dihydrothebainol analog) has shown mild analgesic activity.

Significance to the program of the Institute: (a) The search for new drugs (whether synthetic or modified naturally occurring ones) capable of controlling severe clinical pain, with a minimum of untoward side-effects, properly falls within the purview of the Institute program.

(b) The delineation of a hitherto unknown chemical structure is always scientifically significant.

Proposed course of project: In order to enhance (analgesic and/or muscle relaxing) activity, routes will be explored to (a) reform the 4,5-oxide bridge in the dihydrothebainone analog and (b) demethylate the 3-methoxyl group as well as the N-methyl group.

Part B included: Yes.

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Individual Project Report
Calendar Year 1958

Part B.

Publications:

Sargent, L. J., Schwartzman, L. H. and Smith, L. L.: Hydroxylated acetamide derivatives. J. Org. Chem.: 23: 1247-1251, 1958.

Sargent, L. J. and Agre, J. Sanderman: Carbinolamines derived from 3-(2-bromoacetyl-N-acetyl asym-octahydrotriazidine. J. Org. Chem. Soc. 19[illegible]

PHS-NIH
Individual Project Report
Calendar Year 1958

Serial No. NIAMD 36

1. Laboratory of Chemistry
2. Analgesics
3. Bethesda

Part A.

Project title: Chemical structure and action of morphine-like analgesics.

Principal Investigator: Nathan B. Eddy

Man years: Total : 3 1/3
Professional : 2/3
Other : 2 2/3

Project description:

A. Research:

The screening program of potentially analgesic compounds, prepared here and in other laboratories, has proceeded at a good pace, though the number of compounds examined was a little less than in 1957. Analgesic activity has been stepped up tremendously, on the one hand, and practically eliminated on the other hand, with variable changes in other morphine-like effects. It is now known that analgesic activity can be very greatly increased, in animal screening, confirmed also in man, without corresponding increase in physical dependence potency, in fact with simultaneous diminution in this latter property. Patents have been applied for on N.I.H. 7519, the best example to date of the dissociation of effects just referred to. See also project report of Dr. May for further pharmacological details.

B. Consultative:

Section, largely but not exclusively through its chief, continues to play a very active role in the whole field of development of analgesic agents and the study of addiction. The Section is a clearing house for information, often the center for distribution of coded products for double-blind studies and in some instances coordinator of collected data. (See list of publications).

Work on the Addiction Information Center (to be known in the future as "Classified Information on Narcotics" has begun. As this progresses the Section will be in an ever better position to answer questions on morphine-like substances with respect to both activity and addiction liability.

Part B included: Yes.

PHS-NIH
Individual Project Report
Calendar Year 1958

Part B:

Publications:

Eddy, Nathan B., Halbach, Helwig, and Pellmont, Bella: Studies in analgesics. XV. Aralkyl substitution on nitrogen of morphinan. United Nations Bulletin on Narcotics. In press.

Eddy, Nathan B., and Lee, Lyndon E. Jr.: The analgesic equivalence to morphine and relative side action liability of oxymorphone (14-hydroxydihydromorphinone, Numorphan). J. Pharmacol. In press.

Eddy, Nathan B., Lee, Lyndon E. Jr., and Harris, Carl A.: The rate of development of physical dependence and tolerance to analgesic drugs in patients with chronic pain. I. Comparison of Morphine, Oxymorphone and Anileridine. United Nations Bulletin on Narcotics. In press. A condensation of this paper will be published in the Bulletin of the World Health Organization.

Honors and Awards:

From April 29 to May 24, 1958, member of U. S. delegation to U. N. Commission on Narcotic Drugs, Thirteenth Session, Geneva, Switzerland.

Sept. 29-Oct. 1, and again Dec. 8-10, participated in conferences on current research at Addiction Research Center, Lexington, Ky.

Oct. 6-11, participated as Chairman of ninth session of Expert Committee on Addiction-Producing Drugs, World Health Organization at Geneva, Switzerland.

Nominated by invitation of the Secretary of State of the U. S. as participating scientist in the opium research programme established by resolution of the Economic and Social Council, Nov. 27, 1958.

Serial No. NIAMD 37

1. Chemistry
2. Analgesics
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project title:

1) Optical resolution of (±)-2'-[ ]oxy-2,5,9-trimethyl-6,7-benzomorphan (NIH 7410) and the corresponding N-phenethyl compound (NIH 7519).

2) Isolation and proof of structure of a di-stereoisomer of NIH 7...

3) Some derivatives of 1-hydroxy-2-naphthoic acid.

4) A further attempt to synthesize 5-(m-hydroxyphenyl)-2-methyl-2-azabicyclo [4.3.1] decane.

Principal Investigator: Everette L. May.

Other Investigator: J. Harrison Ager

Cooperating Units: None

Man years: Total : 2
Professional: 1
Other : 1

Project description:

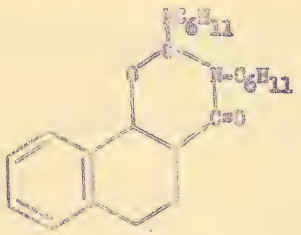
Objectives: Synthesis of a specific analgesic and/or tranquilizing agent and performance of basic research in chemical structure - neuropharmacologic action relationships as well as in synthetic and degradative organic chemistry.

Methods employed: The optical resolution of the synthetic racemic (±)-2'-hydroxy-2,5,9-trimethyl-6,7-benzomorphan (Ia) has been achieved in good yield with (+)-α-bromocamphor-π-sulfonic acid. Each antipode was then converted to the corresponding N-phenethyl compound (Ib) by a sequence involving N-demethylation of Ia, phenylethylation of the resulting secondary amine and $LiAlH_4$ reduction of the amide. An alternate synthesis of N-phenethyl [illegible] has been accomplished in this manner.

Ia (R = CH_3)
Ib (R = $CH_2CH_2C_6H_5$)

2) During the course of large-scale synthesis of NIH 7410 (Ia) not only was an improved procedure developed but two by-products were isolated in 1-2% yields. One of these proved to be 2-(p-hydroxybenzyl)-1,3,4-trimethylpiperidine while the other has been shown to be a diastereoisomer (at C_9) with Ia by degradation of each to 7-methoxy-1,2-dimethylnaphthalene via exhaustive methylation and palladised charcoal aromatization.

3) In some preliminary attempts to synthesize amides of 1-hydroxy-2-naphthoic acid (in analogy with the 3-phenylsalicylamides) using N,N'-dicyclohexylcarbodiimide there was isolated a product whose IR spectrum, analytical and other data are consistant with the structure (II), a naphtho-1,3-oxazine derivative.



II

4) Further attempts have been made to synthesize 6-(m-hydroxyphenyl)-2-methyl-2^{9}-azabicyclo [3.3.1] nonane, a homolog of NIH 7215 (comparable to morphine in analgesic potency). Although we were successful in preparing the methoxy analog in better overall yield than hitherto, o-demethylation has not yet been successful.

Major findings: The (-)-isomer corresponding to Ia proved to be at least twice as potent as the racemate and to be of low toxicity, superior to morphine in both respects (in mice). The (+)-isomer was inactive at a sub-toxic dose. Thus a remarkable separation of activity and toxicity has been achieved. The (-)-isomer of Ib proved also to be twice as potent as the (±)-form, 10 times more potent than morphine. However, the (+)-Ib was also surprisingly effective (1/3 as potent as morphine). The two enantiomers are of about equal acute toxicity. A study of the physical dependence liability of all four isomers is being made for comparison with the racemates.

The diastereoisomer of 7410 (Ia) isolated in small yield from the residues of the 7410 synthesis proved to be 7 times as potent as 7410, 5 times more than morphine. Perhaps it should give an isomer ten times as effective as morphine while appropriate substitution on the nitrogen can be expected to produce compounds 100 times morphine in potency. Experiments are underway to provide an alternative synthesis for this diastereoisomer. Furthermore, based on our research of the

past year and of previous years it can be postulated with reasonable certainty that the introduction of a hydroxyl group meta to the quaternary carbon of an analgesic containing an aliphatic tertiary nitrogen 2 carbons removed from the quaternary carbon, markedly lowers or practically abolishes analgesic activity. The reverse is true when the nitrogen is harmocyclically bound as in Ia.

Cooperating laboratories: [illegible] None : French. Use complementary to pharmacological investigations in this section.

Significance to the program of the Institute: Basic and applied research in the area of neuropharmacologic drugs is perhaps more significant to the program and aims of NIMH than NIAMD but can most certainly be reconciled with our overall program aimed at combating metabolic diseases in which pain and anxiety states are bound to be prominent.

Proposed course of project: We are pursuing research designed 1) to improve or circumvent difficult steps in the chemical synthesis of potentially useful drugs uncovered by us in the last few years; 2) to show what structural changes might be expected to improve neuropharmacologic behavior; 3) to explain why this is so, and 4) to develop improved neuropharmacologic agents.

Part B included: Yes.

Part B.

Publications:

May, E. L.: Structure related to morphine. I. A partial synthesis of racemorphan. J. Org. Chem. 23: 947-949, 1958.

Small, L. F., Eddy, [illegible] and May, E. L.: An improved synthesis of 3-phenethylpiperidine and analogs. J. Org. Chem.: 23: 1387-1388, 1958.

Fry, E. M. and May, E. L.: Morphan derivatives of analgesic activity. J. Org. Chem. 23: 1390, 1958.

Honors & awards:

Appointed to World Health Organization Advisory Panel on Addiction Producing Drugs for a term of five years.

Presented lecture "Synthetic Analgesics" to Merck & Co. Laboratories in March 1958.

Presented "Synthetic Analgesics" to Smith Kline & French Laboratories in May 1958.

Presented "Synthetic Analgesics" to the Chemistry Department of the University of Maryland in June 1958.

Serial No. NIAMD 39
[illegible]
[illegible]
3. Bethesda

Individual Project Report
Calendar Year 1958

Part A:

Project title: Synthesis of [illegible] related to morphine.

Principal Investigator: Edward M. Fry

Other Investigators: None

Cooperative Units: None

Man years: Total : 1
Professional: 1
Other : 0

Project description:

Objectives: Synthesis of compounds to be tested for analgesic activity and isolation of a stimulant particle which arises in the course of a benzomorphan synthesis.

Methods employed: Standard methods of organic chemistry as well as the more recent technic using nuclear magnetic resonance spectra supplied by the Laboratory of Physical Biology.

Major findings: a) The attachment of 3-phenyl-3-oxo-N-propyl residue to the nitrogen of normorphine gave a compound with an encouraging suppression of physical dependence liability and analgesic effect, another example of this sort of separation described by Dr. Eddy, which may be of practical value.

b) Investigation of the Grewe synthesis with the objective of creating a 5-membered B-ring is currently under way.

Significance to the program of the Institute: All work is pertinent to the program as stated in the project title.

Proposed course of project: A continuation of the present investigation.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1958

Part 3

Publications:

Dry, E. K. and Mary Peacock [illegible]. Mannich Derivatives of Analgesic Agents. J. Org. Chem. In press.

Individual Project Report
Calendar Year 1958

NIAMD 39
1 Laboratory of Chemistry
2 Analgesics
3 Bethesda

Part I.

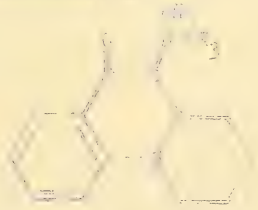
Project title: Synthetic Analgesics; Simpler Morphine Analogs.

Principal Investigator: James G. Murphy
Other Investigators: None
Man years:

Total	:	1.0
Professional	:	1.0
Other	:	0.0

Project description:

Aminohydrophenanthrenes - Certain aspects involved in the stereochemistry of this group of compounds have been clarified. A way has been found to the synthesis of 10 dimethylamino-cis-1,2,3,4,4a,10a-hexahydro-9(10) phenanthrenone. I.



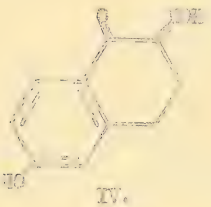
I.

Hydronaphthalenes - In connection with the question whether or not analgesic activity can reside in simpler ring analogs consideration has been given to the synthesis of certain 6-hydroxy-1,2,3,4-tetrahydro-naphthalenes, II.

II

To this end, an improved preparation of the naphthol ether III has been developed from the corresponding naphthol methyl. Certain modifications

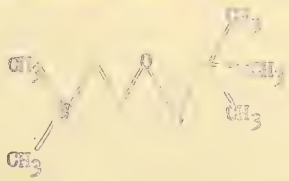
III



IV.

of the usual acid catalyzed isomerization gave IV in good yield. Intercatalytic reduction of the isonitroso group followed by reduction-hydrogenolysis at C-3 was not successful. (Lobeline sensation side reaction?) but it is expected that gentle mercury stirring or removal of the 1-oxo group to permit easy access to II ($R_1 = R_2 = H$).

Hindered Choline Analogs - In view of the role of acetylcholine in nerve transmission (it is also present in sensory nerves), of the penetrability of nerve tissue to tertiary amines and not to the corresponding quaternary ammonium compounds, of the inherent analgesic activity of dimethylaminoethanol, V, the evaluation of sterically hindered esters of V as neuromuscular agents, acting via reversible enzyme inhibition was proposed, and the first of these substances, dimethylaminoethyl pivalate, VI, is now at hand



Significance to the program of the Institute: Implementation of the programmed research on analgesics

Proposed course of project: Development along the lines indicated.

Part II included: No.

Individual [illegible]
Calendar [illegible]

NIMH 40
Laboratory of Chemistry
Analg[illegible]
Bethesda

Part A.

Project title: Comparison of [illegible] and loss of tolerance to the effect of morphine on an analgesic (hot-plate) response and a general behavioral response (swimming) in the rat.

Principal Investigators: Joseph Cochin and Conan Kornetsky.

Other investigators: None

Cooperating Units: NIMH

Man Years: Total : 1
Professional: 2/3
Other : 1/3

Project Description:

Objectives: To determine whether the rate of tolerance development on the one hand, and loss on the other hand is different for two different effects of narcotic drugs.

Methods and Results: The response of the rat after a test dose of morphine to the analgesic effect is measured by using a hot-plate similar to that used by N. B. Eddy for the mouse, and at the same time the effect on speed of swimming a circular alley is also measured. These observations were made before, during and after a seventy day period of twice-daily morphine administration up to a maximum daily dosage of 200 mg/kg. Although an earlier experiment using the tail-flick response to radiant heat as the analgesic measure had shown faster development and loss of tolerance to the analgesic response to morphine than to the effect of morphine on swimming, the results of these later studies seem to indicate that tolerance to the analgesic (as measured by the hot-plate) develops more rapidly and is lost much more slowly than tolerance to the effect on swimming. Three months after abrupt withdrawal, the tolerance to the effect on swimming is almost completely gone, but the analgesic response is only forty per cent of the initial pre-addiction value.

The differences in the results of the two studies may be a reflection of the differences in the drug responses or they may indicate a possibility that we have been measuring three parameters rather than two; the tail-flick which is mainly a spinal reflex response; the hot-plate response, which is more of a total animal response, and the swimming response which is a generalized behavioral response.

Significance to the program of the Institute: Contribution to the understanding of the mode of action of analgesic drugs.

Proposed course of the project: It is hoped to continue the experiment now in progress until recovery of the two parameters we are measuring is complete and thus gain some information as to the time required for recovery from loss of sensitivity to narcotics. We also hope to broaden these studies and to see whether hypo and hyper responders to morphine are also hypo and hyper responders to other centrally-acting drugs.

Part D included: No.

NIAMD 41

1. Laboratory of Chemistry
2. Analgesics
3. Biochemistry

Part A.

Project title: Biochemical and [illegible] Changes after Chronic Administration of [illegible] Drugs.

Principal Investigator: Joseph Cochin

Other Investigator: Julius Axelrod (NIMH)

Cooperating Unit: NIMH

Man years: Total : 1/3
Professional: 1/3
Other :

Project description:

Objective: a) To determine whether suppression of N-demethylation of narcotic drug substrates by liver microsomes after chronic administration of narcotic drugs parallels diminution of in vivo drug response and whether enzyme in the N-demethylase can serve as a model for in vivo changes with tolerance.

b) To investigate the nature of this enzyme diminution.

Methods and Results: a) Using the diminution of analgesic response to a test dose of morphine as the in vivo measure and N-demethylation as the in vitro measure of change, it was found that short-term (two-week) administration of nalorphine, normorphine, morphine and morphine-nalorphine mixtures resulted in a diminution of both parameters which was qualitatively similar. Those drugs which depressed N-demethylase also induced cross-tolerance to morphine and the degree of demethylase diminution seemed to be a rough index of the degree of cross-tolerance induced by a drug. Both nalorphine and normorphine produced cross-tolerance to morphine. In comparing the results by short-term administration to those of long-term (10 days) administration done previously, it was found that the total amount of drug given per day rather than the number of days of drug administration determined both the degree of tolerance and the degree of enzyme diminution.

We were also able to show presumptive diminution of both parameters after just two days of administration of morphine in dosages of 15 mg/kg twice daily. In studying the recovery of enzyme activity and of drug responsivity in animals given morphine for two weeks and then abruptly withdrawn, preliminary experiments indicate that the enzyme activity is fully recovered within 10-14 days, while the analgesic response after a test-dose of morphine is but half of the initial response before chron-

drug treatment.

(b) In studying the mechanism of the demethylase depression, a heat-stable, dialyzable cofactor has been found for this microsomal enzyme reaction. The nature of this cofactor and its properties is now under investigation.

Significance to the program of the Institute: We believe this to be a contribution to the understanding of the mechanism of tolerance.

Proposed course of projects: We expect to continue these studies of N-demethylase recovery and development of tolerance under various experimental conditions. We also propose a study the tolerance-producing capabilities of "narcotic" analgesic drugs such as dextromoramide which other workers have shown to have little or no effect on demethylase activity.

Part B included: Yes.

Part B.

Publications:

Cochin, J. and Axelrod, J.: Biochemical and pharmacological changes in the rat following chronic administration of morphine, nalorphine and normorphine. J. P. E. T. In press.

Honors and awards:

Presented seminar for the Laboratory of Clinical Science, NIMH, on "Biochemical & pharmacological changes in tolerance". November 14, 1958.

Presented lecture to Clinical Associates of NCI on "Clinical uses of narcotic drugs", October 20, 1958.

Serial No. NIAMD 42
1. Chemistry
2. Analgesics
3. Bethanidine

Individual Project Report
Calendar Year 1958

Part I.

Project Title: Clinical evaluation of analgesic compounds.

Principal Investigator: Joseph Cochin

Other Investigator: Nathan B. Eddy

Cooperating Units: None

Man Years: Total : 1/3
Professional: 1/3
Other :

Project description:

The work on substances related to morphine as potential analgesics with little or no addiction liability moved at a very slow pace. In spite of constant review of the patient census on that part of it to which we have access, very few patients have become available to us. The only definitive correlation recorded is that N-cyclohydroxyphenethyl is too strong a morphine antagonist and precipitates severe abstinence syndromes even in patients sufficient to morphine. Its study has been abandoned.

Part II included: No.

Serial No. NIAMD - 43
1. Chemistry
2. Steroids
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Study of Fecal Steroids

Principal Investigator: Erich Heftmann

Other Investigators: Eichio Sato, Harold K. Miller, Rhinehard Weiss and Erich Mosettig

Cooperating Units: None

Man Years (calendar year 1958)
Total: 2-1/3
Professional: 1-2/3
Other: 2/3

Project Description:

Objective.-- To identify steroids in feces.

Methods Employed.-- Adsorption and partition chromatography, preparation of derivatives and infrared spectroscopy.

Major Findings.-- In addition to coprosterol and cholesterol, lithocholic acid, isolithocholic acid, and 12-ketolithocholic acid have been isolated in crystalline form.

Significance.-- Only desoxycholic acid has so far been isolated from human stool, although cholic acid is also known to be present. This is the first reported isolation of lithocholic acid, isolithocholic acid, and 12-ketolithocholic acid from human stool. Lithocholic acid also occurs in human bile, but isolithocholic acid and 12-ketolithocholic acid must be products of the intestinal flora.

Part B included Yes

NIH - NIAMD
Individual Project Report
Calendar Year 1958

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Mosettig, E., Hoffmann, K., Sato, I., and Moore, E., "Occurrence of Lithocholic Acid in the Feces of Healthy Men," Science, 128, 1433-4 (1958).

Moore, J. A., and Hoffmann, E., "Chemistry of the Adrenocortical Hormones," in Handbuch der experimentellen Pharmakologie, Springer Verlag, Berlin - accepted for publication.

Publications concerning report for calendar year 1957, but not reported there:

Zorbach, W. W. and Payne, T. A., Jr., "2-Deoxy Sugars. I. 3,4-Di-O-p-nitrobenzoyl-1-chloro (and 1-Bromo)-1,2,6-trideoxy-D-ribo-hexoses. Two Crystalline 2-Deoxy Acylglycosyl Halides," J. Am. Chem. Soc., 80, 3564-68 (1958).

Zorbach, W. W., "Concerning the Synthesis of Methyl (11-Deoxycorticosteron-21-yl 2,3,4-tri-O-acetyl-β-D-Glucoside) Uronate," J. Org. Chem., 23, 1797-99 (1958).

Serial No. NIAMD - 45
1. Chemistry
2. Steroids
3. Bethesda

PHS--NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: A Study of the Steroidal Alkaloids and Sapogenins

Principal Investigator: Yoshio Sato

Other Investigators: George Lathan (left 8/13/58) and Nobuo Ikekawa

Cooperating Units: None

Man Years (calendar year 1958):
Total: 2-2/3
Professional: 1-2/3
Other: 1

Project Description:

Objective.-- With the objective of finding new, rare and fruitful sources for the production of biologically active steroids, the examination of the degradative possibilities of various steroidal alkaloids and sapogenins have been continued.

In view of the wide spread occurrence of the solanum species, the plant source of solasodine, our attention was directed toward the degradation of solasodine which would lead to an increase in the yield of 5,16-pregnadien-3β-ol-20-one, the conventional source material for the production of progesterone and other biologically active hormones.

Methods Employed.-- In the course of our experiments toward the above objective, we have found that the treatment of steroidal alkaloids with a solution of zinc chloride-acetic anhydride-acetic acid leads to an unsaturated pseudo derivative which may be of use for further conversion into progesterone. Studies are being continued along these lines. It was also found that the sapogenins as a class are converted into an unsaturated derivative whose structure is being elucidated at the present time. This unique reaction is not only of academic interest but may prove to have some industrial value.

TIS--NIH
Individual Project Report
Calendar Year 1958

Major findings.-- A new [illegible] derivative of the steroidal alkaloids whose double bond present in the F ring can be shifted over to the A(5,6) position so as to be amenable for oxidation leading to pregnadienolone.

Significance.-- Possibility of utilization of solasodine and tomatidine as a commercial source of steroids.

Part B included No

Serial No. NIAMD - 45
1. Chemistry
2. Steroids
3. Bethesda

PHS--NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: The Structure of Cholegenin and Isocholegenin

Principal Investigator: Malcolm M. Thompson

Other Investigator: Erich Mosettig

Cooperating Units: None

Man Years (calendar year 1958)
Total: 2
Professional: 1-1/3
Other: 2/3

Project Description:

Objective.-- Structural studies on cholegenin, isocholegenin and dihydrocholegenin were completed.

Method Employed.-- A four-step synthesis of the would-be dehydrocholegenin 16,22-epoxyfurostane-3α,26,27-triol was completed and found to be nonidentical with dihydrocholegenin. Further degradative studies and infrared analyses led to the elucidation of the structures of the cholegenins.

Major Findings.-- It was shown that the structure of cholegenin and isocholegenin is not as reported in the literature by Masur and Spring. The structure of dihydrocholegenin was established to be a 16,22-epoxyfurostane-3α,25,26-triol. Cholegenin is shown to be a 5β,22,25-spoxyfurostane-2α,3β-diol, and isocholegenin a 5β,22,25-epoxyfurostane-3β,25-diol.

Significance.-- (a) The occurrence of the only known steroidal sapogenin containing a five-membered F-ring and being at the same time a neopentyl alcohol in the animal organism is unique from a biogenetic point of view.
(b) Gives an added insight into the mechanism of 3-25 isomerism in steroidal sapogenins.

Part B included Yes

PHS - NIH
Individual Project Report
Calendar Year 1958

Part B. Honors, Awards, and Publications

Publication concerning research for calendar year 1957, but not reported there:

Gupta, I. S. and Mosettig, E., "Identification of β-Sitosterol in Chaulmoogra Oil," J. Indian Chem. Soc., 35, 210-211 (1958).

Serial No. NIAMD - 46
1. Chemistry
2. Steroids
3. Bethesda

PHS--NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Total Synthesis of Anthrasteroids

Principal Investigator: Erich Mosettig

Other Investigators: Alfred Burgstahler (left 9/1/58)

Cooperating Units: None

Man Years (calendar year 1958)
Total: 1
Professional: 1/3
Other: 2/3

Project Description:

Methods Employed:-- Conventional synthesis.

Major Findings.-- The D-form of a 17β-ol-anthrasteroid has been completed.

Significance -- Confirmation of structure of anthrasteroids (Mos-Mosettig), possibility of preparing fully unsaturated anthrasteroids with a short 17-alkyl group (for carcinogenic testing).

Part B included No

Serial No. NIAM - 47
1. Chemistry
2. Steroids
3. Bethesda

PHS—NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Irradiation of 7-Dehydrocholesterol and Ergosterol

Principal Investigator: Osamu Tanaka

Other Investigators: Irish Bossetti and William R. Nes (left 5/30/58)

Cooperating Units: None

Man Year (calendar year 1958)
Total: 2
Professional: 1
Other: 1

Project Description:

Objective.-- This study was undertaken to isolate hydroxylated anthrasteroids obtained from irradiation of some $\Delta^{5},7$-steroids as previously reported by Tsuda and Hayatsu.

Methods Employed.-- The irradiations were carried out in benzene-ethanol mixture in the presence of mercuric acetate, p-toluenesulfonic acid and in an atmosphere of nitrogen. The irradiation source was two G8-sunlamps and one GE H4-1 lamp. The irradiation products were isolated by chromatography.

Major Findings.-- (a) The 7-dehydrocholesterol irradiated product was 7-dehydrocholesterol peroxide. (b) The ergosterol irradiated product was ergosterol peroxide.

Significance.-- Preliminary studies of the irradiated products of 7-dehydrocholesterol and ergosterol show only their respective peroxides as isolatable crystalline products. The melting points and rotations of the peroxides found in this investigation are possibly identical with those reported by Tsuda and Hayatsu for their hydroxylated anthrasteroids.

Part B included Yes

PHS - NIH
Individual Project Report
Calendar Year 1958

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Nes, W. R., "A New Method for the Activation of Copper Chromium Oxide as a Reducing Catalyst," J. Org. Chem. 22, 899-900 (1958).

Nes, W. R., Steele, J. A. and Mosettig, E., "The Anthrasteroid Rearrangement. V. The Preparation of an Analog of Progesterone," J. Am. Chem. Soc. 80, 5230-32 (1958).

Nes, W. R., Steele, J. A. and Mosettig, E., "The Anthrasteroid Rearrangement. VI. The Preparation of an Analog of the Androgens and Estrogens," J. Am. Chem. Soc. 80, 5233-35 (1958).

Serial No. NIAMD - 48
1. Chemistry
2. Steroids
3. Bethesda

Individual Project Report
Calendar Year 1958

Part A.

Project Title: The Partial Synthesis of Sulfur-analogs (Positions 11 and/or 9) of Hydroxylated Corticoids and Other Steroidal Hormones

Principal Investigator: Toshio Kawasaki

Other Investigators: Erich Mosettig

Cooperating Units: Cancer Chemotherapy National Service Center, NCI -

Man Year (calendar year 1958)

Total: 1-2/3
Professional: 1
Other: 2/3

Project Description:

Objective.— This study has been commenced with the objective of finding antimetabolites of corticoids and steroidal sex hormones. Such antimetabolites may shed light upon the mechanism of action of antirheumatoid and cancerchemotherapeutic steroidal agents.

Methods Employed.— The methods employed consist mainly (a) in the adding of thiocetic acid or trichlorothioacetic acid to the 9,11-double bond; (b) in the opening of the 9,11β-, 9,11α-, or 11,12β-epoxide ring with appropriate reagent (hydrogenthiocyanide, thiocetic acid, trichlorothioacetic acid, hydrogen sulfide or methyl mercaptane).

Major Findings.— The 9,11-double bond resists addition to form 9- or 11-substituted compound. The 9,11α-epoxide ring resists opening and conversion to 9-hydroxy-11β-substituted compound by conventional methods. The 9,11β- or 11,12β-epoxide ring is opened and converted by hydrogenthiocyanide to 11β-hydroxy-9α or 12α-thiocyano steroid. The 9,11α-epoxide in

Individual Project Report
Calendar Year 1958

5β (A/B cis) series seems to be converted to 11β-thiocyano-3,9α-epoxide.

9α-Thiocyano series, 4-thiocyano-hydrocortisone (and their acetates) Δ⁴-androsten-9α-thiocyano-11β-ol-3,17-dione and Δ¹-androsta-9α-thiocyano-3,11,17-trione were prepared.

Part B included No

Serial No. NIAMD - 62
1. Chemistry
2. Steroids
3. Bethesda

[illegible]
Individual Project Report
Calendar Year 1958

Part A.

Project Title: The Structure of the Aglycone of Steviosid[illegible]

Principal Investigator: Hans [illegible] Lichti (left 8/3/58)

Other Investigator: Erich Mosettig

Cooperating Units: None

Man Years (calendar year 1958)
Total: 1
Professional: 1
Other: None

Project Description:

Objective.— To determine the final structure of the aglycone.

Major Findings.— The previously proposed rearrangement of steviol to isosteviol has been confirmed by a number of elucidative reactions. Further attempts were made at the structural proof of the Wagner-Meerwein rearrangement of ring A.

Significance.— There appears to be still a strong industrial interest in utilizing steviosid as a natural sweetening agent.

Part B included No

Serial No. NIAMD - 50
1. Chemistry
2. Steroids
3. Bethesda

PHS--NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Bile Acids from Human Feces

Principal Investigator: Yoshio Sato

Other Investigators: Erich Hoffmann and Ekkehard Weiss

Cooperating Units: None

Man Years (calendar year 1958)
Total: 1
Professional: 1/3
Other: 2/3

Project Description:

Objective:-- To isolate and identify new bile acids as well as other acidic substances from the acidic fraction of human feces.

Method Employed.-- The acidic fraction of pooled human feces was subjected to a multifold purification process - countercurrent distribution, adsorption and partition chromatography, crystallization.

Major Findings.-- Lithocholic acid has been isolated for the first time from human feces. Another unsaturated bile acid has been isolated. Work is being continued toward the identification of this substance as well as isolation of other hitherto undiscovered acids from human feces.

Significance.-- The isolation and identification of bile acids from human feces is important in that it may shed some light on the possible pathways of their metabolism. Bile acids from human feces have been a relatively neglected field.

Part B included No

Serial No. NIAMD - 51
1. Chemistry
2. Steroids
3. Bethesda

PHS - 2 7
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Excretion of Adrenocortical Hormones in Urine

Principal Investigator: David F. Johnson

Other Investigators: Erich Heftmann

Cooperating Units: Walter Reed Army Medical Center - supplies pregnancy urines

Man Years (calendar year 1958)
Total: 2-1/3
Professional: 1-1/3
Other: 1

Project Description:

Objective.-- Study of corticosteroid excretion in pregnancy.

Methods Employed.-- The urinary extracts are fractionated on partition columns with water as the stationary phase. Gradient elution with petroleum ether containing increasing amounts of dichloromethane gives fractions of the individual adrenocortical hormones. The fractions are analyzed by ultraviolet absorption and reduction of blue tetrazolium. Their identity is established by paper chromatography.

Major Findings.-- Sixteen major compounds found in the urinary excretion pattern of pregnant women have been investigated by means of paper chromatography. Two of these have been identified as 3α,17α,21-trihydroxypregnane-11,20-dione (tetrahydro S) and 3α,11β,17α,21-tetrahydroxypregnan-20-one (tetrahydro F). The remaining compounds have been characterized as to chromatographic behavior, number of oxygen functions, and side chain at the 17-position of the steroid nucleus. The evidence to date indicates that these compounds may be metabolites of the known active adrenal hormones involving in vivo reduction at the 3,4,5- and 20-positions of the steroid nucleus. The quantitative excretion data of these individual

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Individual Project Report
Calendar Year 1958

compounds in the serum of normal pregnancy and in certain complications such as toxemia or rheumatoid arthritis show a definite variation that is not apparent in total assay.

Part B included No

Serial No. NIAMD - S2
1. Chemistry
2. Steroids
3. Bethesda

PHS--NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Infrared and Ultraviolet Spectroscopic Studies

Principal Investigator: Harold K. Miller

Other Investigator: Mrs. Anne H. Wright

Cooperating Units: None

Man Years (calendar year 1958)
Total: 2
Professional: 1
Other: 1

Project Description:

Approximately 1080 spectra were made to support investigations in progress in the Steroid Section. The infrared micro illuminator was used to investigate metabolic steroidal compounds from urine. The inability of the separation procedures employed to rid micro samples of impurities still hampers the ultimate helpfulness which might otherwise be expected for this technique. Attempts to correlate infrared spectra of morphine and related compounds are being continued as time permits. The Beckman prism-grating spectrophotometer (IR-7) has been utilized to locate N-H stretching vibrations in tomatidine and solanodine, heretofore not observed, using the sodium chloride prism and usual concentration conditions. An investigation of difference spectra in the infrared region of cholesterol, Δ^5-cholestene, and dehydroepiandrosterone indicates that it may be feasible to conveniently identify side chain character by this technique. An investigation along these lines is continuing.

Part B included No

Serial No. NIAMD - 93
1. Chemistry
2. Steroids
3. Bethesda

PHS--NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Editorial Service

Principal Investigator: Charles I. Wright

Other Investigator: None

Cooperating Units: None

Man Years (calendar year 1958)
Total: 1
Professional: 1
Other: None

Project Description:

As Chairman of the Editorial Committee of this Institute, I received and recorded 36 manuscripts (ranging from books to abstracts) since November 1, 1957. Each manuscript was usually assigned to two or more members of the Committee, including the Chairman, for review. When no board member qualified the Chairman sought and received aid from members of the other Institutes or occasionally from persons in the National Bureau of Standards and the Naval Hospital Research Laboratories. The evaluation of each reviewer's comments was made by the Chairman, especially when a manuscript was not recommended for publication or severely criticized. A few such manuscripts required consultation with the author and a second or third reading before final approval or rejection.

Part B included No

Serial No. NIAMD- 54
1. Chemistry
2. Metabolites
3. Bethesda

FSS VIII
[illegible] Report
Calendar Year 1958

Part A.

Project Title: Inhibition of the Biosynthesis and Breakdown of 5-Hydroxytryptamine and Other Centrally Active Biogenic Amines

Principal Investigator (at NIAMD): Bernhard Witkop

Other Investigators: M. Ozaki (arrived 5/1/58)

Cooperating Units: S. Udenfriend, E. Weissbach and B. Redfield, Lab. of Clinical Biochem., NHI, Serial No.

Man Years (Calendar year 1958):
Total: 2/3
Professional: 2/3
Other:

Project Description:

Project: Synthesis of compounds which inhibit 5-hydroxytryptophan decarboxylase and monoamine oxidase (MAO).

Objectives: The inhibition of the enzyme which decarboxylates 5-(L)-tryptophan or dihydroxyphenylalanine would prevent the formation of serotonin or dopamine and other catecholamines and have practical applicability in diseases characterized by overproduction of serotonin and catecholamines such as liver cirrhosis syndrome and pheochromocytoma tumor. The inhibition of MAO in vivo and in central locations is known to result in marked psychic effects. The control of both of these processes is highly desirable.

Methods Employed: The technique for assaying MAO oxidase activity is described in the publication by Pretam, Weissbach et al. Similar techniques are being used or investigated by Dr. M. Ozaki working in Dr. Udenfriend's laboratory for the decarboxylases acting on dopa, tryptophan and 5-hydroxytryptophan, as well as on catechol-O-methyltransferase.

Major Findings: One of the most powerful MAO inhibitors was discovered in the naturally occurring harmala alkaloids, which have been known for a long time and have recently attracted renewed attention as ingredients of plants taken by some natives of the Peruvian Andes to produce psychic effects. Harmaline was studied in this connection and showed in vitro as well as in mice, rats and humans an

activity for greater [illegible] the [illegible] commercially available [illegible] inhibitors such as [illegible] or [illegible]. The action of harmaline is completely reversible in contrast to [illegible]. An extensive [illegible] program which is being carried out by [illegible] has uncovered [illegible] in vitro (in vivo) activity [illegible] a number of well-known drugs such as [illegible] amide (Squibb) and (rthonine (Upjohn).

Significance to the Program of the Institute: The [illegible] of monoxidase inhibitors has acquired so much importance to have a recent session of the New York Academy of Sciences was devoted to it. The exact methods for carrying out inhibitors are being used in the laboratory of Dr. Udenfriend. In order to support these important investigations Dr. Osuki of [illegible] necessary has been delegated to participate in a program of developing and screening inhibitors in this area. Many pharmaceutical companies supply compounds for this program. They also adopt these enzymatic screening methods in their research programs.

Proposed Course of Project: Attempts will be made to isolate harmala alkaloids which will be more readily absorbed from the gastrointestinal tract. Reversible inhibitors of MAO and various [illegible] bases will be looked for. The program of clinical cooperation will be expanded.

Part 2 included: Yes

Part B [illegible], Awards [illegible] Publications

Publications other than [illegible] from this project:

Tweten, E., Weissbach, [illegible] Redfield, B., Udenfriend, S., and Witkop, B. Oxidable Analogs of 5-[illegible]-tryptophan as Inhibitors of the Biosynthesis and Breakdown of Serotonin. J. Am. Chem. Soc. 80: 983-987, 1958.

Udenfriend, S., Witkop, B., Redfield, B. G., and Weissbach, H.: [illegible] with Reversible Inhibitors of Monamine Oxidase. Harmaline and Related Compounds. Biochem. Pharmacol. 1: 166, 1958.

Serial No. NIAMD- 55

1. Chemistry
2. Metabolites
3. Bethesda

PHS-1
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Metabolism and Biosynthesis of Catechol Amines

Principal Investigator (at NIAMD): Bernhard Witkop

Other Investigators: Siro Senoh

Cooperating Units: [illegible] and J. R. Creveling, NHI [illegible]
J. Axelrod, NIMH, Serial No.
Dr. Sydney Archer, Sterling-Winthrop Research Institute

Man Years (calendar year 1958):
Total: 1
Professional: 1
Other:

Project Description:

Synthesis of Rare Metabolites of Dopamine, Norepinephrine, Adrenaline and Other Catechols of Physiological Importance. Clarification of the Biosynthesis of Norepinephrine.

Objectives: To establish metabolic parameters for important endogenous hormones, to characterize new enzymatic metabolites, to study labile transformation products of dopamine and precursors of norepinephrine by elucidating the mechanism of its formation.

Methods Employed: Isotope labeling of dopamine with tritium and C^{14} was used to follow the chemical and enzymatic transformations including the conversion to noradrenaline.

Major Findings: The oxidation of nucleophilic reagents such as water or methanol to (3-hydroxy-) 6-hydroxydopamine proceeds from the alkaline and 6-hydroxy- [illegible] 4,5-trihydroxyphenylethylamine) at a ratio of 10,000:1. 4,5,6-trihydroxyphenylethylamine is easily formed by autoxidation from solutions of dopamine on standing, with certain tissues or in the enzymatic tyrosinase system. This new and reactive product of dopamine is characterized chemically and distinguishable from noradrenaline. The discovery was made possible only by cross-labeling technique whereby it was found that the "noradrenaline" fraction had not lost any significant tritium activity. The same technique showed that tissue from selected regions of the brain, such as hypothalamus and caudate nucleus, convert dopamine to authentic noradrenaline in yields up to 4%.

A number of new aminochromes and tetrasubstituted indoles have been prepared [illegible] of dopaminochromes and the mechanism of these transformations has been followed by tritium labeling. It remains to be seen whether these new aminochromes or some of their derivatives are centrally active. Such activity has been claimed without sufficient support for adrenochrome and adrenolutin.

Since it has recently been postulated that dopamine may be a new hormone in certain tissues, it is of interest to note that preliminary studies in the rat indicate that 6 hydroxydopamine, after administration of dopamine to animals.

Collaborative efforts with [illegible] Wenthrop aim at the synthesis of amino acid precursors of [illegible] of pyramidine, the blood-brain barrier and of the release of active amines such as adrenaline, noradrenaline etc. in the brain. It was hoped that [illegible] in the National Institute of Mental Health that these biogenic amines when labeled and given intravenously to animals did not reach the brain.

Some of these results have been presented at the [illegible] Chicago, Sept. 7-12, 1958, [illegible] Abstracts p. 6NP, and at the IV International Congress of Biochemistry, Vienna, Sept. 1-6, 1958.

Significance to the program of the Institute: The metabolic fate of [illegible] and centrally active biogenic amines is a key problem in modern neurochemistry and psychopharmacology.

Proposed [illegible] of the project: Studies will continue on the conversion of dopamine to [illegible]. Methods will be developed for the diagnosis and [illegible] of 3,4,5-trihydroxyphenylethylamine, and its in vivo significance will be investigated. A major metabolic product from dopamine, [illegible] 3,4-dimethoxyphenylethylamine, will have to be [illegible].

Part I included: Yes

Part D. Honors, Awards, and Publications

Publications other than abstracts from this project:

Senoh, S., and Witkop, B. (NIAMD), and Creveling, C. R., and Udenfriend, S. (NHI): Oxidation Mechanisms of Catecholamines and the Biogenesis of Norepinephrine (Oxidation Mechanisms XII.). Contribution to the Colloquium on Oxygenating Enzymes (Chairman: Professor O. Hayaishi), 5th International Congress of Biochemistry in Vienna, Sept. 4-6, 1961. Congress Monograph, In Press.

Serial No. NIAMD- 56
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Selective Cleavage of the Hydroxyproline Peptide Bond in Model Peptides and in Collagen

Principal Investigator: Bernhard Witkop

Other Investigators: John Francis (U.S., arrived 11/7/58)

Cooperating Units: H. A. Piez, NIDR, Serial No. _____

Man Years (Calendar year 1958):
Total: 1/3
Professional: 1/3
Other: ---

Project Description:

Objectives: To find a selective chemical method for the cleavage of peptide-bonds next to hydroxyamino acids, especially hydroxyproline with a view of applying such cleavage methods to the structural elucidation of collagen and gelatin.

Methods Employed: It had been observed previously in this laboratory that suitable O-acyl hydroxy-L-proline derivatives, in the presence of proton acceptors, undergo an intramolecular elimination reaction with the formation of allo-hydroxy-L-proline lactones. The principal of this reaction is now being applied to suitable hydroxyproline-peptide derivatives.

Major Findings: The brief period available for this project allowed only a few preliminary experiments which indicate the liberation of a free amino acid from a dipeptide such as N-carbobenzyloxy-O-methylsulfonylhydroxy-L-prolyl-L-phenylalanine ethyl ester.

Significance to the Progress of the Institute: Collagen, the quantitatively most important protein in animals, is characterized by its high content of hydroxyproline, an amino acid occurring more or less exclusively therein. A specific method for the selective cleavage of the hydroxyproline peptide bond is highly desirable, since such enzymes fail to cleave this bond. Such a method would make it easier to find, as has been reported, the areas of high hydroxyproline content in the peptide strands of collagen (Grassmann), and would facilitate the analysis of urinary hydroxyproline peptides observed in some patients with metabolic disturbances (Dr. F. Irreverre).

Part B Publications: None

Serial No. NIAMD- 57
1. Chemistry
2. Metabolites
3. Bethesda

NIH-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: The Chemistry and Metabolic Fate of Tryptamine

Principal Investigator (or NIH): Bernhard Witkop

Other Investigators: John Daly

Cooperating Units: (1) Undefined and Associates, Lab. of Chemical Biochem., NHI, Serial No. _____, Regional Med., Chicago (custom synthesis of certain deuterioindoles)

Man Years (Calendar year 1958):
Total: 1-1/3
Professional: 1
Other: 1/3

Project Description:

Objectives: The discovery of Dr. Udenfriend that tryptamine arises from tryptophan by the action of a new decarboxylase, and that it occurs in significant amounts in the brain, focuses attention on this new biogenic amine and its transformations.

Methods Employed: Non-enzymatic and enzymatic oxidations of cold and radioactive tryptamine lead to a number of new compounds which are under investigation. Nuclear magnetic resonance spectrophotometry is utilized to gain information on the possible existence of unstable tricyclic tautomers of tryptamine.

Major Findings: The appearance of tryptamine in the brain has raised the question of its possible transformation to serotonin. Such a conversion, however, has now been ruled-out, since radioactive tryptamine in vivo does not lead to radioactive serotonin. The nuclear magnetic resonance spectra of tryptamine derivatives leave room for the assumption of a small amount of a labile tricyclic tautomer. Attempts to trap this tautomer in the form of an autoxidation product so far have led to interesting new compounds whose structure is under investigation. Tryptamine in brain, according to S. Udenfriend et al., good but not hydroxytryptamine not identical with serotonin. In analogy to some Japanese findings this new metabolite has been considered as 5-hydroxytryptamine. However, recent results make the 6-hydroxytryptamine more

liminary. Synthetic studies, aided in part by the Regis Chemical Co., [illegible] the final identification of these new hydroxyindoleethylamines.

[illegible] of the Institute: The recent findi[illegible] that the [illegible] used for producing central effects in re[illegible] ligious animals exist in at least several [illegible] forms, [illegible] the O-conjugate of 5-hydroxy-N,N-dimethyltryptamine, [illegible] interest in the more hydroxylated tryptamine metabolites mentioned above.

Irregular Course of Menopause: The chemistry and pathophysiology of 6- and 7-hydroxy-[illegible]oleethylamine will be investigated.

Dept. Int. Med.: [illegible]

Serial No. NIAMD- 58
1. Chemistry
2. Metabolites
3. Bethesda

NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Regulation of Growth of Animal and Plant Cells by Derivatives of Natural Hydroxyamino Acids

Principal Investigator: Bernhard Witkop

Other Investigators: Arthur A. Patchett

Cooperating Units: Dr. F. C. Steward, Cornell University
Dr. C. Mitoma, NHI, Serial No. _____
[illegible], Western Washington Research Institute

Man Years (Calendar year 1958):
Total: 2/3
Professional: 1/3
Other: 1/3

Project Description:

Project: To determine the influence of hydroxyamino acids and their analogues on possible regulations or inhibitions of cellular growth.

Objectives: To accomplish a more direct control of protein synthesis, tissue segregation, formation of collagenous scar tissue by direct and local application of cyto-active agents than was hitherto possible by remote and hormonal control.

Methods Employed: A plant tissue culture system has been used to detect the growth inhibitory effect of a number of nitrogenous compounds and to determine, where possible, the metabolic site at which the substance in question may act. The tissue culture system consisted of explants from carrot root stimulated to grow by cell division. This was suggested because the cells which grow in this way synthesize a protein in which proline is incorporated and which is unusually rich in hydroxyproline for a plant protein.

Major Findings: 1) In these tissues see publication by Steward, Pollard, Patchett and Witkop "The Effects of Selected Nitrogen Compounds on the Growth of Plant Tissue Cultures," Biochimica et Biophysica Acta, 28: 301, 1958). 2) In chicken embryos ketoproline, an analog of hydroxyproline, caused a prolonged elevation of free hydroxyproline. The mechanism for this elevation has been established to be due to inhibition of hydroxyproline oxidation by ketoproline and by enzymatic conversion of ketoproline to hydroxyproline. The enzyme for the latter reaction is found in the supernatant fraction of rat kidney and liver.

and [illegible] pyrrolidine-2-carboxylic acid. Some of these [illegible] have been summarized in the [illegible] International Congress of Biochemistry; Abstracts, Section [illegible], No. 2[illegible], p. 1[illegible].

Significance to the Progress of the Institute: The existence of enzymes capable of effecting hetoproline-to-hydroxyproline raises the question of a possible transformation which, on the level of collagen, could lead to the formation of cross-linkages. Whether the hardening of collagen with age may be due to such a sequence of reactions remains to be seen.

Proposed Course of Project: Dr. [illegible] and [illegible] will continue the effect of proline and [illegible] hetoproline on animals and will try to purify the enzyme involved in the conversion of hetoproline to hydroxyproline. The Fels Biochemical Research Institute is cooperating on the large scale preparation of hetoproline.

Part B included: Yes

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Mitoma, C., Smith, [illegible], Udenfriend, S., [illegible], A. A., and Witkop, B.: Studies on 4-Keto-L-proline. Science, In Press.

Part I

Project Title: Studies on [illegible] and on the Chemistry of Neuro-Muscular Blocking Agents

Principal Investigator (at NIH): Bernhard Witkop

Other Investigators: G. Holland (left 2/28/58), H. Kay

Cooperating Units: S. Friess, Naval Medical Center

Man Years (Calendar year 1958):
Total: 1
Professional: 2/3
Other: 1/3

Project Description:

Objectives: To establish [illegible] possibly of use for detection of those more and dissimilar in the manner as building stones of antibiotics; to explore the steric limitations and requirements for (polyfunctional) substitution of choline esters; to correlate neuromuscular blocking activity with the presence or cyclic analogs of dimethyl carbamoyl choline.

Major findings: The hydrolysis of ester bonds in para-O-esters of tertiary and quaternary 2-dialkylamino-ethyl and acyclic-acetal, as well as of tetrahydrofurfuryl esters in the strychnidine series, have been correlated with the empirical classification of their hydrolytic cyclohexane analogues. In order to find the limitations and rates of this kind of inhibition the cyclonic of the analogous derivatives of the deoxystrychnidine series was studied by methylation of deoxystrychnine, available by the hydrolysis of brucine (accepatin A) from which to yield the diketone base which by reduction of acetic anhydride under mild and strong conditions unexpectedly gave only diacetates, which by kinetic assay proved to be a mixture of a hydrolytically inert and active species. Only 15% of a labile diacetate was obtained admixed with 85% of the inert diacetate. The hydrolytic activities of these labile esters approached the reactivity of esters as labile as p-nitrophenyl acetates.

The anticholinesterase activity of synthetic D,L-muscarine and 9 further derivatives was determined with a highly purified enzyme preparation from electric eel tissue by a constant pH titration technique used previously (S. L. Friess, I. B. Isbell and B. Witkop, J. Am. Chem. Soc. 73: 659, 1957). A proportionality between the activity thus by muscarine was

exhibited by trans-[illegible] dihydrochloride, dl-a-acetyl-β-methyl-muscarine. These compounds exhibited parallel pharmacological findings.

Certain features of the inhibition of the system acetylcholine-esterase-acetylcholine by the cis and trans isomers of 2-hydroxycyclohexyltrimethyl ammonium iodide have been studied and their effect on the relative substrate levels employed. At pH 7.4 and 25° with a protein concentration of the order of 2×10^{-4} g. per ml. inhibition by each of these isomers is apparently competitive at substrate levels of 1.5×10^{-3} M and lower, but is found to deviate from the competitive relation at higher levels. A possible model to account for this behavior as well as the feature of inhibition of the system by excess substrate has been discussed.

Significance to the Program of the Institute: Two major aspects of the cholinesterase problem are of special interest. 1) the mode of hydrolysis and nature of ester linkage cleavage. 2) The correlation of structure and activity in natural and synthetic inhibitors.

Further Course of Project: Dr. [illegible] is presently occupied with pharmacological and biochemical investigations involving inhibition of cholinesterase. It will be of special interest to construct inhibitors capable of penetrating the blood brain barrier and of acting on brain cholinesterase directly.

Part B included: Yes

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Mautner, H. G., Jones, S. L., and Withrop, B.: Two Acetylcholine-esterase Surfaces. II. Dependence of Competitive Inhibition by Diquinuocyclobutane Derivatives on Substrate Level. J. Am. Chem. Soc. 80: 5687-5692, 1958.

Holland, G. F., Withrop, B., and Friess, S. L.: Labilization of Water Bonds in Aminoacetal Quaternary Derivatives as Mediated by Nitrogen Conformation. Experientia 14: 129-130, 1958.

Holland, G. F., Durant, R. C., Friess, S. L., and Withrop, B.: Labilization of Water Bonds in Aminocyclitol Derivatives. I. Derivatives of Myo and Scyllo Inositols and of Streptamine. J. Am. Chem. Soc. 80: 6031, 1958.

Serial No. NIAMD- 60
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Selective Cleavage of Peptide Bonds by Chemical Agents

Principal Investigator: B. Witkop

Other Investigators: A. Patchornik (U.S., left 10/29/58), J. B. Lawson, Erhard Gross (U.S., started 10/2/58)

Cooperating Units: None

Man Years (calendar year 1958):
Total: 2-2/3
Professional: 2-1/3
Other: 1/3

Project Description:

Objectives: 1. To investigate and apply methods for the selective cleavage of peptide bonds. 2. To selectively modify or cleave peptides and enzymes in order to correlate structural elements with physiological or enzymatic activity.

Methods Employed: Specific oxidizing agents, such as 2-hydroxy-5-bromosuccinimide, were capable of selectively attacking, e.g., tryptophan residues in peptides and proteins. In situ observation of the changes in absorption by differential ultraviolet spectroscopy, using a split-recording instrument devised to follow this reaction. Ehrlich and Stein and Moore's techniques, paper chromatography and electrophoresis serve for the identification of cleavage products.

Major Findings: The ultraviolet absorption of tryptophan-containing proteins is accounted by characteristic shifts in the ultraviolet spectrum to shorter wavelengths. Similar shifts are also produced by the controlled oxidation of, e.g., N-(benzoyl)-tryptophan, glycyl-tryptophan, lysozyme, trypsin and other proteins with oxidants, such as N-bromosuccinimide in water. The reaction was followed in situ by differential UV spectrophotometry and proceeded smoothly in ~ 10^{-3} molar solution. After the rapid consumption of approximately 1.5 equivalents of oxygen the indole chromophor had disappeared. Volatile titration of the reaction mixture showed the presence of only 20% of ammonia for pairing to indole bromination accompanying oxidation of the indole ring to derivatives of oxindole. Model studies with skatol, indole-β-propionic acid and N-benzoyl-tryptophan confirmed this observation. In a parallel

scale indole-β-propionic acid rapidly consumed three moles of [illegible] aqueous alcoholic acetate buffer at pH 4. From the reaction mixture a lactone (m.p. 80-85°) was obtained. Apparently, the very labile bromine atom in the β-position of the oxindole ring is displaced by an internal SN2 reaction by the carboxylate anion of the side chain. This neighboring group effect of a potentially nucleophilic amide imidol group in an indole β-amide chain was utilized for the cleavage of the N-peptide bond adjacent to tryptophan. Whereas N-Cbz-tryptophylglycine methyl ester gave rise to glycine, the isomeric N-Cbz-glycyltryptophan under these conditions did not liberate any amino acid. The general usefulness of the method was demonstrated with [illegible] the crystalline hydrazide-glycine-glycylglycine peptide from [illegible] containing only the tryptophan among 4N amino acids. N-bromosuccinimide leads to the [illegible] of a yellow monohydrin-quinone, giving positive ninhydrin-chloride reactions for methionine and tryptophan reactions for histidine and arginine. Its hydrolysis yields tryptophan, acid, threonine, a dibasic acid and lysine. This tetrapeptide which arises from the [illegible] of ACTH-(1-24)-NH2, has been obtained by the action of glycyltrypsin on trypsin in glucagon. However, the cleavage of glucagon by N-bromosuccinimide is more rapid (<1 min.) and more selective than that by any known peptidase.

Significance of the project: There is a great need for more selective methods for the controlled enzymatic degradation of proteins. These specific "peptidases" in many ways promise to be superior to all known enzymes most commonly used for breakdown and structural investigation of proteins.

Further Course of Project: It is to be expected that the new cleavage reaction will find wide application among biochemists. In order to speed up progress on the study and limitations of these methods collaborative experiments are being planned on the following projects: 1) Dr. [illegible] will investigate with Dr. Gladner [illegible] of Physical Biology Section on Fluorine Biochemistry, to what extent chymotrypsinogen can be unfolded with urea without cleavage and will no longer be activated to chymotrypsin. 2) Dr. Gross [illegible] spend some time in Dr. [illegible] laboratory at the Weizmann Institute to find out whether he can detect any physiological activity of a number of cyclic antibiotic peptides. 3) Dr. L. K. Ramachandran, from the [illegible] laboratory of the University of California, it is hoped, will spend some time in this laboratory to look at the selective cleavage of the protein [illegible] peptide [illegible] especially of the fraction having the molecular weight of [illegible]. Dr. T. Viswanatha is planning to [illegible] enzymatically [illegible] active fragment (m.w. 5,000) from [illegible] which contains only [illegible] tryptophan. The analysis of the [illegible] points to the location of lysine. It is expected [illegible] to furnish clues on the amino acid sequence of the active center in trypsin.

Part B included: Yes

Part B. Honors, Awards, or Additional Items

Publications other than Journals from this project:

Patchornik, A., Lewis, W. B., and Witkop, B.: The Use of Intramolecular Group Effects for the Selective Cleavage of Peptide Bonds. I. On the Mechanism of Oxidation of L-Substituted Indoles with N-Bromosuccinimide. J. Am. Chem. Soc. 80: 4748, 1958.

Patchornik, A., Lewis, W. B., and Witkop, B.: Selective Cleavage of Peptide Bonds. II. The Tryptophyl Peptide Bond and the Sequence of Glucagon. J. Am. Chem. Soc. 80: 4747, 1958.

Nat'l. Inst. of Health
1. Chemistry
2. Laboratories
3. Bethesda

1958-61

Individual Project Report
(Calendar Year 1958

Part A.

Project Title: Biochemical Chemistry of Enzymatic Degradation of Heterocyclic Compounds of Medical Importance (Imidazole Derivatives)

Principal Investigator: Bernhard Witkop

Other Investigators: H. Kny (V.S.), T. W. Beiler, V.S., July 1958, regularly: Stetson Univ.

Cooperating Units: None

Man Years (Calendar year 1958):
Total: 2/3
Professional: 2/3
Other:

Project Description:

Objectives: In the enzymatic degradation of imidazolone-5-propionic acid to formiminoglutamic acid one intermediate has been postulated to be 4(5H)-imidazolone-5(4)-acrylic acid. This compound is extremely labile and undergoes a number of rearrangements which are under study. In particular the attempt to establish the biological imidazolonepropionic acid is between histidine and formiminoglutamic acid.

Major Findings: Imidazoleacrylic acid was prepared by controlled intramolecular cyclization of 2-acetylaminoacrylic acid and was found to have a half-lifetime of close to one hour around pH 8. A purified enzyme preparation from Pseudomonas caused the disappearance of the characteristic band at 299 mμ significantly more rapidly than the spontaneous reaction. The enzymatic cleavage however requires ammonia as also shown by the lack of effect of inhibitors such as versene and cyanide, whereas p-chloromercuribenzoic acid inhibited 100% at 10⁻⁵ molar. A great number of intermediates were observed and identified by semi-quantitative paper chromatography. The enzymatically imidazolonepropionic acid cleaves at the "enamide" imidine bond to give formiminoglutamic acid, a non-enzymatic hydrolytic degradation seems to be excluded, the highly unstable 4(5H)-hydroxy-6-pyrimidone-4-carboxylic acid which rearranges rapidly to formylaminosuccinimide. Of many approaches to the synthesis of imidazolonepropionic acid only the reduction and diazotization of nitroimidazolepropionic acid proved feasible. At least 4 products arise

this reaction will be studied. Some of these findings have been presented at the [illegible] International Congress of Biochemistry, Sept. 1958, cf. Abstracts 3-26, p. 173.

Proposed Course of Project: The nature of the lability of 4-imidazolones will be further investigated, especially the mechanism of the formation of these unique derivatives which must lead through the hydropyrimidones whose labilities surpass somewhat those of the known dihydrouracil, dihydrothymine, dihydroorotic acid etc. The conversion of histidine, free and in peptides, to imidazolones will be investigated with the aim of elucidating methods for the selective cleavage of histidyl peptide bonds. The chemistry of 4(5H)-imidazolone-5(4)-propionic acid, when available, will be studied since it has direct bearing on this problem.

Significance to the Progress of the Institute: The labile acyl-amidine bond, the existence of 5,6-dihydro-4-pyrimidones, the formation of 4(5H)-imidazolones are all unusual under a series of metabolic interest. The selective cleavage of histidyl peptides would be of great importance to protein chemistry.

Part B included: No

Serial No. NIAMD- 62

1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Nuclear Magnetic Resonance Spectra of Indole Derivatives.

Principal Investigator: Louis A. Cohen

Other Investigators: J. W. Daly, H. Kay, B. Witkop

Cooperating Units: Laboratory of Physical Biology, Section on Photobiology, NIAMD 109

Man Years:
Total: 1/3
Professional: 1/3
Other:

Project Description:

A study of characteristic features in the NMR spectra of various indole derivatives of biochemical significance.

Objectives: [illegible] facilitating the [illegible] investigate the [illegible] ethylamines.

Methods Employed: [illegible] isolated from natural sources, labile hydrogens exchanged with deuterium and the [illegible] for correlative data.

Major Finding: It has been possible to determine the absence or presence of α and β substitution in indoles of considerable complexity by means of NMR spectra. The spectra of tryptamine and its analogs suggest [illegible] detection.

Significance: The ability to determine the nature and degree of α and β substitution in indoles [illegible] determination of new natural indole derivatives (reserpine, etc.) [illegible] of metabolic products of tryptophane (serotonin, tryptamine, chief-

nating to a large degree on elaborate chemical degradations. The existence of ring-chain tautomerism in indole-2-ethylamines may provide an insight into the mode of action and the high degree of specificity of serotonin, ecarine, tryptamine and similar indole derivatives.

Future Course of Project: To prepare and examine the uv spectra of various substituted tryptamines. The discovery of indole derivatives with extensive cyclic character may provide serotonin analogs with a higher degree of physiological activity.

Part B included: No

Serial No. NIAMD- 63

1. Chemistry
2. Metabolites
3. Bethesda

NIH-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: The Synthesis and Biochemical Activity of Certain Sequences of Insulin

Principal Investigator: Louis A. Cohen

Other Investigators: Gerald F. Holland

Cooperating Units: None

Man Years:
Total: 1/3
Professional: 1/3
Other:

Project Description:

To synthesize and study the enzymatic properties of certain cyclic disulfide fragments of insulin.

Objectives: To determine the relative importance of geometrical factors vs. chemical constitution in the hormonal action of insulin and in its sensitivity to insulinase.

Major Findings: A new method for the synthesis of sulfur-containing peptides has been developed which permits the preparation of peptides with several cysteine residues and the specific manipulation of one sulfhydryl at a time.

Significance: Since the variation in certain amino acids in the insulin molecule does not alter the hormonal activity, it is essential to know whether the over-all geometry alone is sufficient to impart biological activity. In the hope of contributing to this problem as well as to an understanding of the mechanism of insulin action, this project has been undertaken.

Future Course of Project: To complete the synthesis of the desired cyclic peptides and to study their behavior in enzymatic and physiological systems.

Part B included: Yes

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

Holland, G. F. and Cohen, J. S. Studies on the Synthesis of Insulin Peptides. J. Am. Chem. Soc. 80: 3765-3769, 1958.

Serial No. NIAMD-64

1. Chemistry
2. Metabolites
3. [illegible]

[illegible]-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: A [illegible] for the synthesis of substituted thyronines and thyroxine analogs

Principal Investigator: Louis A. Cohen

Other Investigators: None

Cooperating Units: None

Man Years:
Total 2/3
Professional 1/3
Other: 1/3

Project Description:

The synthesis of [illegible] by [illegible] diaryl ethers by means of [illegible] dienone intermediates.

Objectives: To convert a series of 3,5-dihalo-4-hydroxy-benzoic acids to the corresponding benzilimines, to effect a condensation of these with substituted phenols and to remove the carboxyl function and aromatize ring A by decarboxylation.

Methods: Ultraviolet spectroscopy is used to follow the course of the dienone-phenol condensation as well as decarboxylation. Infrared spectroscopy is used to elucidate structures of the reaction products.

Major Findings: Methyl 3,5-dibromo-4-hydroxybenzoate has been converted to its [illegible] by N-bromosuccinimide. The dienone underwent condensation with 2,6-dimethoxyphenol to form [illegible] dienones.

Significance: The variety of thyronine analogs available for study has been severely limited by the synthetic methods existing for their preparation. With the development of the synthesis devised herein, the variety of derivatives available for testing should be greatly increased. The method would also make available some of the unstable intermediates proposed for the biosynthesis of thyroxine.

Future Cour[illegible] prepare for test[illegible] unavailable.

Part B included: No

Serial No. NIAMD-6S

1. Chemistry
2. Metabolites
3. Bethesda

[illegible]-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: [illegible] Acid-Base Catalysis of Decarboxylation: A Possible Enzyme Model.

Principal Investigator: Louis A. Cohen

Other Investigators: None

Cooperating Units: None

Man Years:
Total: 2/3
Professional: 1/3
Other: 1/3

Project Description:

To study the mechanism of decarboxylation of p-substituted benzoic acids and related compounds under relatively mild conditions of pH.

Objectives: To show that comparatively acid base catalyzed decarboxylation can serve as a model for enzyme systems utilizing the same principle of action.

Methods Employed: The use of ultraviolet spectroscopy to measure the kinetics of a series of reactions.

Major Findings: p-Nitrobenzoylaminoacetic acid undergoes decarboxylation at reflux by buffer catalyzed mechanisms with maxima at pH 4.5 and pH 9. The reaction at pH 9 is thought to proceed via the quinomethide acid in one of the rare evidences for such intermediates in the reactions of phenolic compounds.

Significance: The present study is structurally similar to pyruvic acid, [illegible] and oxalic acid and, particularly to the pyridoxal-amino acid complexes. The information gained in this study may further an understanding of the enzymatic decarboxylation of these systems.

[redacted] to test the catalytic activity of internal "push-pull" catalysts such as 2-hydroxypyridine.

Part B included: No

Serial No. NIAMD-66

1. Chemistry
2. Metabolites
3. Bethesda

NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: [illegible] a specific chemical peptidase

Principal Investigator: Louis A. Cohen

Other Investigators: G. L. Schmir

Cooperating Units: None

Man Years:
Total: 1
Professional:
Other:

Project Description:

A study of the chemical fragmentation of polypeptides and proteins at tyrosyl-peptide bonds by use of oxidizing agents such as bromine and N-bromosuccinimide.

Objectives: To effect the splitting of complex peptides by the addition of a chemical reagent which attacks tyrosine moiety and inhibits the adjacent peptide bond. To study the use of phloretic acid as a unique and highly specific amine blocking group in peptide synthesis.

Methods Employed: Rapid recording ultraviolet spectroscopy is used to follow the rates and extent of cleavage reactions. Infrared spectroscopy is used to elucidate the structure of reaction products. Paper and column chromatography and high-voltage electrophoresis are used to separate and purify polypeptide fragments.

Major Findings: Peptides and proteins containing tyrosyl-residues are split exclusively at the tyrosyl-carboxyl bond with the liberation of the latter as free amino acid. The tyrosine fragment is changed to a non-acidic dicarbone-lactone.

Significance: The ability to split complex peptides (enzymes and proteins) at specific bonds can contribute greatly to the determination of amino acid sequences, to the modification of proteins without denaturation and to the isolation of active fragments of enzymes. By these techniques proteins can be split under very mild

conditions (buffer, pH, organic solvent) by rapid acyl shift reaction at selected position.

Future Course: To determine the optimum conditions for selective cleavage of threonyl-peptide bonds and to extend the study to more complex systems.

Part B included: No

Serial No. NIAMD- 67

1. [illegible]
2. Independent Unit
3. Bethesda

[illegible]
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Analytical Services Laboratory

Principal Investigator: William C. Alford

Other Investigators: [illegible] Paulson, Evelyn G. [illegible] Lyon, Elizabeth H. Fath, Victor E. Hart, William R. Jones (to September 1, 1958).

Cooperating Units: [illegible] Section on Liver Diseases, NIH, NIAMD & Section on Polysaccharides, LCP, NCI

Man Years:
Total: 7 1/3
Professional: [illegible] 2/3
Other: 1 2/3

Project Description:

The Analytical Services Laboratory is a service organization providing servi[illegible] for research personnel of the National Institutes of Health, and [illegible] limited extent, for persons of other governmental agencies. The scope of this work is summarized as follows:

1. Approximately 9000 elemental, functional group and instrumental analyses were performed during the past year. These, with the approximate numbers of each indicated, were as follows: carbon (200), hydrogen (200), nitrogen by Dumas, Kjeldahl, Pregl, and ultramicro techniques (2[illegible]), reducing sugars (1[illegible]), halogens (2[illegible]), sulfur (60), phosphorus (300), functional groups such as acetyl, methoxyl, benzoyl, acetonyl, [illegible] hydrogen (150), [illegible] Karl Fischer-solvate-cast etc. (1[illegible]), [illegible] weighings (200), [illegible] such as sulfate, oxygen, ash, chromium [illegible], calcium etc. (60), optical rotations (10), infrared and ultraviolet spectra (750), and [illegible] (3[illegible]), miscellaneous (250). Recipients of this service include about 125 research workers of the NIH staff. In addition, small [illegible] personnel on governmental agencies outside NIH, where such service can be released without interfering with the progress of NIH research. During this year such outside service was rendered chiefly to persons at the Naval Medical Research Institute, Bethesda, Md., and the Food and Drug Administration, Washington, D. C.

2. During the year the staff of the Analytical Services Laboratory has provided services of a special nature to two separate research teams, namely, that of Dr. Klaus Schwarz (NIAMD) and of Dr. Peter More (NHL). The former involves a study of the dietary importance of selenium while the latter is concerned with the anti-coagulant properties of various polysaccharides. The selenium study has been of such magnitude as to require the full time efforts of one member of the staff. Selenium determinations are tedious and time-consuming and require frequent checks of accuracy. Dr. Schwarz has repeatedly stated that he is pleased with the services rendered. This work is continuing for an indefinite period.

3. Considerable time is spent in consultation with research workers concerning problems which they wish to handle in their own laboratories. Methods of approach are discussed, advice is given and in some cases equipment is provided. For example a complete Warburg unit was recently loaned to Dr. James A. Hattey of the Cancer Institute to permit immediate progress on their research.

4. Plans are being formulated to initiate a new service for which there has been a continuing demand, namely, the direct determination of oxygen in organic compounds. This service can be offered when equipment is procured and a position is allocated to provide for another analyst.

Part B included: No

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

ANNUAL PROJECT REPORT

CALENDAR YEAR 1958

Summary Sheet

LABORATORY OF PATHOLOGY AND HISTOCHEMISTRY

Estimated Obligations for FY 1959

Total - $655,922
Direct - $398,800
Reimbursement - $257,122

Project Numbers 68 thru 82 Included

Individual Project Report
Calendar Year 1958

Serial No. NIAMD 66
1. Pathology & Histochemistry
2. Histochemistry
3. Bethesda

Part I

Project Title: Histochemical studies of connective tissue components

Principal Investigators: R. D. Lillie and G. G. Glenner*

Other Investigators : None

Cooperating Units : NIDR: H & P: Dr. M. S. Burstone
NIDR: H & P: Dr. H. M. Fullmer
NHI: LCB : Dr. H. Weissbach et al.
NCI:E : Dr. H. J. Levine

Man Years (calendar year 1958)

Total: 5
Professional: 2
Others: 3

* Dr. G. G. Glenner, six months at Johns Hopkins on advanced residency training program and last six months at NIH

Project Description:

Studies on enterochromaffin have progressed to the point where some information as to its chemical structure can be deduced. That it is a phenol has of course been apparent since 1930 when Gaudier and Lison demonstrated its alkaline azo coupling capacity. It was presumed at that time and for many years after that, that it was an orthodiphenol on the basis of the chromaffin reaction and a suggestive ferric chloride test.

Comori (1941) ruled out this structure on the basis that the rapidity of ammoniacal silver reduction was slower than that of a catechol. This was confirmed by demonstrating that acid silver nitrate is promptly reduced by catechol and by the reduced phase of melanin, but not by enterochromaffin. Gomori suggested a meta-diphenol structure which certain azo coupling colors tend to support. However, resorcinol and phloroglucinol both exhibit keto tautomerism and it should be possible to prevent the azo reaction by adequate treatment

with sulfite, phenylhydrazine or cyanide. The azo reaction was maintained unimpaired after treatment considered adequate to completely block the reactivity of tissue aldehydes toward Schiff's sulfite fuchsin reagent.

Moreover, a reversible oxidation blockade of the reducing and azo reactions was demonstrated. A number of oxidants, as well as nitrous acid, were successful, and reactivity could be restored by hydrosulfite or amide. These findings suggested a p-diphenol quinone or p-aminophenol quinoneimine relationship, rather than a secondary amine 5-nitrosamine relationship, since other oxidants performed equally well as nitrous acid.

The existence of a primary aryl amine was long since ruled out, perhaps on inadequate evidence, by Lison and Clara. However, our experiments with tyrosine diazotization indicates that not only does enterochromaffin possess no primary arylamine group, but that further we are not able to demonstrate O-nitrosation with subsequent rearrangement to a diazonium nitrate, such as occurs with tyrosine.

Recent metal chelation experiments have demonstrated a definite color change in the azo complexes, especially when Fe^{++} chelation was reinforced by a following ferricyanide reaction. This suggested that the azo coupling was ortho to the directing hydroxyl rather than para. However, parallel chelation experiments on unazocoupled enterochromaffin using iron salts were also successful when followed by color reinforcement with ferricyanide.

Both ferric and ferrous salts on prolonged exposure demonstrate enterochromaffin cells selectively in blue with ferricyanide as the final demonstrating agent. This indicates direct chelation of Fe^{++} and reduction of Fe^{+++} to Fe^{++} with in situ chelation. We were already aware that Fe^{+++} is reduced to Fe^{++} by enterochromaffin. This finding led us back to some type of o-dioxy structure, not an unsubstituted catechol, but possibly an o-methoxyphenol, such as has recently been suggested in the metabolism of the physiological catecholamines.

This hypothesis will be further explored.

Meanwhile exploration of the azo coupling and o-diphenol condensation reactions of enterochromaffin have been under more detailed exploration. Differences in the azo coupling rate with various diazonium salts have been found to be much greater than had been anticipated. It is intended to employ these data in chemical model experiments with various substances exhibiting the suspected structures as well as some claimed in recent literature.

Two methods for the histochemical demonstration of tyrosine have been developed which utilize both diazotization and nitrosation of tyrosine to develop an azo dye and a metal chelate respectively. A modification of the tyrosine methods for application to electron microscopy is being evaluated. A critical investigation of present methods for the histochemical demonstration of tyrosine and tryptophan is being made in an attempt to determine the specificity of these techniques, the limit of their applicability and their relative sensitivity.

Part B included. Yes.

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

R. D. Lillie: The Nile blue reaction of peptic gland zymogen granules: the effect of methylation and alkali demethylation. J. Histochem. Cytochem. 6: 130-132, 1958.

R. D. Lillie: Symposium: Problems of fixation in histochemistry. Opening remarks. J. Histochem. Cytochem. 6: 301-302, 1958.

R. D. Lillie: Acetylation and nitrosation of tissue amines in histochemistry. 6:352-362, 1958.

H. M. Fullmer and R. D. Lillie: The oxytalan fiber: A previously undescribed connective tissue fiber. 6:425-430, 1958. (In Press J. Histochem. Cytochem.)

H. J. Levine and G. C. Glenner: Observations on tryptophan staining of the pancreatic cells. J. Cancer Inst. 20: 63-68, 1958.

G. G. Glenner and M. S. Burstone: Esterase and phosphatase activity in Necturus maculosus. A study in comparative histoenzymology. Anat. Rec. 130: 243-251, 1958.

R. D. Lillie, H. Weissbach and G. G. Glenner: On the experimental discharge of the gastric and intestinal argentaffin cells. (In Press) J. Histochem. Cytochem.

Honors and Awards relating to this project:

Dr. R. D. Lillie was elected President of the Histochemical Society for 1957-1958.

Dr. R. D. Lillie received the "Sustaining Membership Award" awarded by the Association of Military Surgeons of the United States in 1958, in recognition of his many contributions to medicine through his work in experimental pathology over a period of thirty-seven years with the United States Public Health Service. (November 19, 1958)

PHS-NIH
Individual Project Report
Calendar Year 1958

Serial No. [illegible]
1. [illegible]
2. Histochemistry
3. Bethesda

Part A

Project Title: Studies on the histochemical reaction of mucoproteins

Principal Investigators: S. S. Spicer

Other Investigators: None

Cooperating Units: None

Man Years (Calendar year 1958)

Total:	2-1/2
Professional:	1
Others:	1-1/2

Project description:

The work of the past year has concerned primarily the development of histochemical procedures for differentiation of specific tissue constituents. A method considered to offer increased specificity and reproducibility has been devised for staining ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). This procedure depends on acid methylation of tissue sections as a means of differentially blocking (esterifying or hydrolyzing) acid moieties other than nucleic acids and simultaneously hydrolyzing ribosidic linkages in DNA. A Schiff (i.e. Feulgen) stain specific for the liberated deoxyaldoses followed by Azure A staining for the phosphoric acid residues under appropriate conditions, then yields red stained DNA and blue RNA.

A method for staining the proteins associated with DNA rather than the acid groups has been developed, based on the excess of lysine in nucleohistone. This procedure involves staining with acid dyes at pH levels below the pK value of the lysine amine group but above the association level of most other basic groups in tissue sections. The nuclear metachromasia obtained depends in part on the chemical structure of the acid dye, and completion of this study awaits further testing of chemical variants of the dyes thus far employed successfully.

The use of Ehrlich's reagent (N,N-dimethylaminobenzaldehyde; for colorimetric determination of the chromogenic constituent of mucopolysaccharide (sialic acid) suggested the possibility of a histochemical procedure for this substance. Although the conditions for the above determination are not suitable for application to tissue sections, it has been found that sites rich in sialic acid react chromogenically following aldehyde production in the tissue by periodate oxidation and azomethine condensation in acid alcohol medium with the amino analogue of Ehrlich's reagent (N,N-dimethyl-p-phenylenediamine). This reaction also imparts bright orange fluorescence in ultraviolet light to structures colored red. The specificity of the reaction is under investigation but progress has been impeded by lack of availability of authentic standards for testing. An aqueous variant of the procedure gives somewhat different results staining neutral mucopolysaccharides various shades of orange brown depending on species and organ, acid mucopolysaccharides other than those with sulfate esters, pink or purple grey, and sulfate ester containing polysaccharides, black.

Other studies near completion concern application of the histochemical procedures to problems in pathology and histology. Changes in aged mice have been investigated in which there is excessive accumulation of stainable ferric compounds, both intra epithelial and in phagocytes throughout the body. These are associated with deposits of lipofuscins and with increased infiltration of mast cells. Application of the mucopolysaccharide procedure has shown a sex difference in structure of the mouse sublingual glands and possibly in the mucin content of the brush border or the renal tubules.

Part B included.

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

S. S. Spicer & J. H. Bryant: Systemic effects in rabbits receiving injections of papain and chondroitin sulfate. Am. J. Path. 34: 61-75, 1958.

S. S. Spicer: Histological localization of glycogen in the urinary tract and lung. J. Histochem. Cytochem. 6:52-60, 1958.

Honors and Awards relating to this project: None

PHS-NIH
Individual Project Report
Calendar Year 1958

Serial No. NIAMD 70
1. [illegible]
2. Histochemistry
3. Bethesda

Part A

Project Title: Studies on renal structure and function through the use of histochemical techniques

Principal Investigator: J. B. Longley

Other Investigators: None

Cooperating Units:

NCI-LP:	Dr. W. G. Banfield
NIH-LKEM:	Dr. N. Levinsky
NIMH-CI:	Dr. N. Lassen
	Dr. G. W. Still and R. Abdu, George Washington Medical School

Man Years (Calendar Year 1958)

Total:	2-1/2
Professional:	1
Other:	1-1/2

Project Description:

1) Studies of the structure and function of the vascular bundles of the renal medulla have been pursued on several fronts.

a) The effectiveness of these structures as barriers to the entry of diffusible substances into the renal papilla has been demonstrated through the use of suitable tracers. Early in the year kidney autographs from rats injected with I^{131} labelled human serum albumin showed that this protein, to which capillary walls are relatively impermeable, rapidly enters the renal papilla in large amounts. By contrast, highly diffusible I^{131}-labelled antipyrine was found to enter relatively slowly, and never to exceed the concentration achieved almost instantly in the cortex. These results were confirmed quantatively by studying the simultaneous incorporation into the papilla at short time intervals of tracer systems composed of one diffusible and one nondiffusible substance, each labelled with an isotope having a substantially different half life. Colloidal Au^{198}-I^{131}-antipyrine was one such system, I^{131}-human serum albumin-$Na^{24}Cl$ another. The results obtained in this way indicate that the non-diffusible components enter the papilla three or more times as fast as the diffusible. (With Dr. Lassen, NIMH-CI).

b) Although the foregoing indicates the operation of an effective countercurrent exclusion, the potential efficiency of such structures in biological form can approach 100% (as in the "rete gland"

of the swimbladder of fishes). Since sodium actually enters the papilla at a readily appreciable rate, attempts were made to determine whether the source of the sodium might not be the urine entering the papilla via the loops of Henle and the collecting ducts. Experiments similar to those above were carried out on animals with ligated ureters. The effectiveness of this procedure in stopping tubular urine flow was proven by demonstrating the failure of I^{131}-insulin to enter the proximal tubules of animals so treated. The results indicate that relative to nondiffusible materials, stoppage of tubular urine flow does not appreciably retard the entry of sodium into the renal papilla. (With Dr. Stoll and R. Abdu, George Washington Medical School).

c) Studies of the fine structure of the medullary vascular bundles have begun. The endothelium of the afferent vessels has been found to be composed of cells having abundant cytoplasm (2-3μ thick) which overlie each other extensively. The efferent vessels have a more typical endothelium about 1000 Å thick. Continued study of this material is necessary to permit interpretation of further details observed. (With Dr. Banfield, NCI-LP).

3) In an effort to interpret the swelling behavior of slices of kidney tissue in various solutions to the behavior of particular structural components, studies have been made of experimental tissues by means of the freeze-substitution method of Feder and Sidman. So far no conclusions have been reached concerning the susceptible elements, but results have suggested more suitable experimental conditions than those so far used. (With Dr. Levinsky, NHI, LKEM).

4) In view of conflicting statements in the literature concerning the distribution of glutaminase I activity in the rat kidney, which appear in part to arise from technical difficulties in separating the several types of tubular tissue in this organ, a study of the question is being made with the benefit of previous experience in the precise localization of kidney enzymes.

Part B included.

Part H: Honors, Awards, and Publications

Publications other than abstracts from this project:

Barnett, E. W., Cole, R. M., May, E. L., and Longley, J. B.: Studies on streptococcal hyaluronidase and antihyaluronidase. II. The localization of sites of absorption of streptococcal hyaluronidase (group C) with fluorescent antibody. J. Histochem. Cytochem. 6: 171-173, 1958.

Lassen, N. A., Longley, J. B., and Lilliehaid, L. S.: Concentration of albumin in renal papilla. Science 128: 720-721, 1958.

Honors and Awards relating to this project: None

Z01 AI No [illegible]
1. Pathology & Histochemistry
2. Pathologic Anatomy
3. Bethesda

PHS-416
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Preparation of stained tissue sections for investigation and diagnostic purpose.

Principal Investigator: Mr. Roy Read - Head, tissue preparation laboratory.

Other Investigator: None

Cooperating Unit: None

Man Years (calendar year 1958):
Total: 7*
Professional: 0
Other: 7

Project Description:

The statistical report of this unit is shown below. In addition to preparing material for projects conducted in the laboratory of Pathology and Histochemistry, many other investigators received advise and service. These include: Drs. Saunders and Kelley of NHI; Drs. Oliver, Dewitt and Rewington - Lab. of Trop. Dis.; Drs. Brodsky, Craighead, Hesselbach and Howe - Lab. Inf. Dis.; Drs. Bauer and Freund - Lab. of Immunology; Dr. Goover - Dental Institute.

Tissues from a small number of animals were prepared for five other investigators.

Polio vaccine studies, control and experimental aspects continued; tissue from 2,227 monkeys were processed for histopathologic study.

* In addition 1 part-time technician furnished by NIS for polio vaccine work.

	Specimens Accessioned	Stained slides Routine	Stained slides Special	Spare Slides	Total
Animals	5,516*	11,712	14,609	17,679	
Surgicals	2,071	4,179			
Autopsies	146	1,421			
					49,670

* Source of animal material:

DBS	2,227
NIAMD	1,776
Other Institutes	1,513

Part B included: No.

Serial No. NIAMD- 72
1. Pathology & Histochemistry
2. Pathologic Anatomy
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Morphologic variations in the reproductive tract and preputial glands of the germ-free and conventional rat, as influenced by hormones, vitamins, and bacteria, and the relationship of these factors to the normal estrous cycle, pseudo-pregnancy, pregnancy, and breeding.

Principal Investigator: David L. Beaver

Other Investigator: Ernest G. McDaniel

Cooperating Unit: LNE, NIAMD. 2

Man Years (calendar year 1958):
Total: ½
Professional: ½
Other: None

Project Description:

The objective of the experiment is to determine, by the use of the germ-free animal and by conventional experimental methods, the relationship of normal bacterial flora to the neutrophilic vaginal exudate of the murine estrous cycle; and to study the eosinophilic infiltration of the rat endometrium in regard to its apparent hormonal variation and possible significance in the reproductive cycle. Concomitantly, the role of vitamin A in the maintenance of the normal cycle and its influence on reproductive function is being studied, specifically in regard to corpus luteum formation and to the decidual reaction. Changes in the preputial gland, which by location at least seems to be an accessory genital organ, are being followed simultaneously. Evaluation of findings at the present time would be premature, but it is hoped that the information obtained will lead to a better understanding of animal breeding, sterility, and reproduction in general.

In addition to the above, I am collaborating with others in an experiment concerned with the production of dietary cirrhosis in the germ-free rat (see report by Dr. L. L. Ashburn).

Part B included: No.

1. Pathology & Histochemistry
2. Pathologic Anatomy
2. Bethesda

NIH-NIH

Individual Project Report
Calendar Year 1958

Part A.

Project Title: Pathology of certain nutritional deficiencies in germ-free rats, allergic encephalitis in guinea pigs, and tumors of the American Indian.

Principal Investigator: L. L. Ashburn

Other Investigators: Ernest G. McDaniel, Floyd S. Daft, David L. Beaver, George A. Hottle and Ruth L. Kirschstein.

Cooperating Units: LNE, NIAMD; D.E.S. 2

Man Years (calendar year 1958):
Total: 3
Professional: 1
Other: 2

Project Description:

Folic acid deficiency - completed studies of a moderate number of animals show that the bone marrow in extremely atrophic in those rats in which anemia is severe and circulating granulocytes markedly depleted, and in other rats roughly correlates with the degree of anemia and agranulocytosis. The bone marrow atrophy in general reaches a greater degree in the germ-free than in the conventional rat. This is probably explained by the fact that these low-stress animals (germ free) can survive on fewer red cells permitting greater duration and severity of their disease. Also liver changes are greater in the germ-free rats and include fatty metamorphosis, at times severe, focal or patchy necrosis and moderate deposition of hemosiderin. The latter is seen in great quantities also in the spleen.

Study of nutritional cirrhosis in the germ-free rat has been started to assess the possible role of intestinal bacteria in this disease. The generally accepted concept that the cirrhosis follows liver cell damage consequent to choline or methionine deficiency has been recently challenged; the claim being that noxious products of intestinal bacteria are responsible for the cirrhosis; the experimental approach used is opened to some question. The germ free should give a clear-cut answer. This should be accomplished in one year.

Rabbies vaccine prepared as suspension of rabbit brain occasionally causes encephalitis in man. Hence all available vaccines of this type (10) were tested in guinea pigs to determine incidence of induced allergic encephalitis. All produced typical lesions of this disease in high incidence. Rabbies vaccine prepared in duck embryo did not produce lesions. Suspension of very young rabbit brains and adjuvants do not cause encephalitis. Further studies will be done to determine the exact age that the rabbit acquires the offending agent.

Study of surgical and autopsy specimens continue on material received from P.H.S. Indian Hospitals and Bureau of Prison Hospitals. This study is performed by various members of laboratory staff. The "tumor of American Indian" study has been extended one year so as to include a slightly larger series. The paper detailing the findings is in rough draft. The study is with Dr. Ruth L. Kirschstein.

Part B included: No.

Serial No. NIAMD 74
1. Pathology & Histochemistry
2. Pathologic Anatomy
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Studies on catechol amines, high altitude, iodates, trypanosomiasis, and experimental cardiac lesions and glomerulonephritis.

Principal Investigator: Benjamin Highman

Other Investigators: P. B. Altland, H. M. Maling, J. Roshe, S. E. Webster, P. A. Shore, E. J. Tobie, V. H. Cohn Jr., E. C. Thompson, and E. F. Stohlman.

Cooperating Units: LPB, NIAMD (Altland and Thompson) 98
LCP, NHI (Maling, Shore and Cohn)
Surgery Branch, NHI (Roshe)
LPT, NIAMD (Webster and Stohlman) 88
LTD, NIAID (Tobie)

Man Years (calendar year 1958):
Total: 1
Professional: 1
Other: None

Project Description:

Serum transaminase determinations are widely used to aid in the differential diagnosis of coronary occlusion and other diseases in man. Highman, Maling and Thompson have found that a similar elevation in serum transaminases may be produced in dogs by a subcutaneous injection of epinephrine-in-oil (1 mg/kg). Lesser elevations follow an intravenous infusion during a period of 83 minutes of epinephrine (0.55-0.92 mg/kg) or norepinephrine (0.51-0.85 mg/kg). The rise in transaminases (SGO-T and SGP-T) after a subcutaneous injection of adrenalin-in-oil is comparable in magnitude to that produced by coronary ligation in dogs, reaches a peak in about 24 hours, and gradually declines to a normal range within 7 days. During this period there is also a sharp rise in serum alkaline phosphatase. The adrenergic blocking agent Dibenzyline prevented the rise in serum transaminase but not the rise in serum alkaline phosphatase. The greater serum enzyme elevations after a subcutaneous injection may be due to slower

absorption and more prolonged action of the drug, since a similarly marked elevation occurred when an intravenous infusion of epinephrine was given more slowly. This study suggests that some of the effects of catechol amines may be due to release of certain specific enzymes from cells and may open a new approach in studying the relation between specific hormones and enzymes.

Normal dogs, it was found, may survive an exposure for several hours to a simulated altitude of 38,000 feet, a much higher altitude than that tolerated by man and some species such as the rat. A study with Drs. Altland and Roshe revealed that dogs with aortic insufficiency and other valvular deformities show little or no reduction in tolerance to simulated high altitude. In another study with Dr. Maling, it was shown that dogs with large myocardial infarcts produced by coronary ligation similarly show only a slight reduction in tolerance to a high altitude exposure.

Studies with Drs. Altland and Roshe on the effects of repeated daily exposures to 30,000 feet on normal dogs and on dogs with valvular deformities are nearing completion. Other high altitude studies and studies on experimental endocarditis and glomerulonephritis in dogs with aortic insufficiency are continuing. A study with Dr. Altland has begun to determine the effects of high altitude exposures on chicks.

A report of studies with Dr. Webster and Mr. E. F. Stohlman on the toxicology of potassium and sodium iodates was sent to the Food and Drug Administration. The proposed use of potassium iodate in place of iodide in salt for human consumption was considered in the light of available toxicological data. Tentative use of this material on a trial basis, subject to a subsequent survey, was judged to possess an adequate factor of safety.

A study with E. J. Tobie on rabbits infected with Trypanosoma gambiense was completed. The rabbits, unlike mice, failed to develop central nervous system lesions, comparable to those produced by trypanosomiasis in man, when the infection was prolonged by a series of subcurative treatments with the amino nucleoside of puromycin following succeeding relapses.

In a study with Shore, Cohn, and Maling, it was shown that the average concentration of norepinephrine in the canine myocardium was about 2.7 µg/g in atrial tissue and about 1.3 µg/g throughout the ventricular myocardium. None was found in the endocardium. These findings suggest that adrenergic nerve endings are widespread throughout both atrial and ventricular musculature.

Studies are continuing with Dr. Maling on the effects of coronary artery occlusion on reserpinized dogs and on the effects of catechol amines, monoamine oxidase inhibitors and various adrenergic blocking agents.

Part B included: Yes

Serial No. NIAMD 74

Individual Project Report
Calendar Year 1958

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Highman, Benjamin, Rocho, Joseph and Altland, Paul D.: Endocarditis and Glomerulonephritis in Dogs with Aortic Insufficiency: Production by Simple Bacterial Inoculation and Effect of Cortisone. A.M.A. Arch. Path. 65:388-394, 1958.

Shore, P. A., Cohn, V. H. Jr., Highman, B. and Maling, H. M.: Distribution of Norepinephrine in the Heart. Nature 181:848-849, 1958.

Tobie, E. J. and Highman, B.: Failure to Demonstrate Cerebral Lesions in Rabbits Infected with Trypanosoma Gambiense. J. Parasitol. 44:273, 1958.

Maling, Harriet M. and Highman, Benjamin: Exaggerated Ventricular Arrhythmias and Myocardial Fatty Changes After Large Doses of Norepinephrine and Epinephrine in Unanesthetized Dogs. Am. J. Physiol. 194: 590-596, 1958.

Maling, H. M. and Highman, B.: High Altitude Tolerance of Normal Dogs and Dogs with Myocardial Infarcts. Am. J. Physiol. In press.

Webster, S. H., Rice, M. E., Highman, B., and Stohlman, E. F.: The Toxicology of Potassium and Sodium Iodates II. Subacute Toxicity of Potassium Iodate in Mice and Guinea Pigs. Toxicology and Applied Pharmacology. In press.

Highman, B., Maling, H. M. and Thompson, E. C.: Serum Transaminase and Alkaline Phosphatase Levels after Large Doses of Norepinephrine and Epinephrine in Dogs. Am. J. Physiol. In press.

Serial No. NIAMD 75
1. Pathology & Histochemistry
2. Pathologic Anatomy
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Experimental studies concerned with central regulatory centers of aldosterone secretion and with the pathogenesis of cardiac lesions in the carcinoid syndrome. Relative incidence of degenerative cardio-vascular diseases and neoplastic diseases in the Japanese.

Principal Investigator: Gert L. Laqueur

Other Investigators: F. C. Bartter; J. O. Davis.

Cooperating Units: CMET, NHI IKEM, NHI

Man Years (calendar year 1958):
Total: 1/3
Professional: 1/3
Other: None.

Project Description:

Autopsy material collected in Japan while attached to the Atomic Bomb Casualty Commission of the NAS-NRC is being organized and reviewed. Tabulation of the material is prepared dealing with such topics as atherosclerosis, coronary heart disease, pathogenesis of cerebro-vascular accidents and certain aspects in oncology. The analysis of the available data should be completed early in 1959.

A study has been initiated dealing with the localization of possible central regulatory centers involved in the mechanism of aldosterone secretion. At present, the structural details of the epithalamus and peri-pineal regions of the brain are being reviewed and restudied in various species. Material previously collected during the study of neurosecretory pathways is being reexamined. Upon completion of these studies which will utilize methods to demonstrate neurosecretory activity of nerve cells as well as certain histochemical methods for localization of enzymatic activity, brains with surgical or electrolytic lesions from animals with known metabolic disturbances will be studied in a similar manner. The second part of the project will be done in close collaboration with members of the laboratory of kidney and electrolyte metabolism of the NHI who will provide basic physiologic data on the experimental animals.

A second project, still in a preparatory phase, is concerned with the pathogenesis of the cardiac lesions seen in cases of "carcinoid tumors" of the small intestine. The endocardial fibrosis which leads to stenosis of the cardiac valves on the right side of the heart has not been reproduced experimentally in spite of many attempts. The possibility exists that the failure to reproduce the heart lesions has been due to the fact that normal animals have been used. Because man shows striking deficiencies in certain amino-acids in this syndrome, it is planned to deplete the experimental animal of these amino-acids prior to its exposure to high serotonin levels. In order to elucidate the peculiar features of the endocardial lesion, thrombosis of blood vessels will be produced experimentally and the organization of the thrombi studied with routine and histochemical methods.

Part B included: Yes

Serial No. NIAMD 75

Individual Project Report
Calendar Year 1958

Part E: Honors, Awards, and Publications

Publications other than abstracts from this project:

Haymaker, Webb, Laquaur, Gert L., Nauta, W. J. H., Pickering, John E., Sloper, John C. and Vogel, F. Stephen: The Effects of Barium^{140}-Lanthanum^{140} (Gamma) Radiation on the Central Nervous System and Pituitary Gland of Macaque Monkeys A Study of 67 Brains and Spinal Cords and 77 Pituitary Glands. J. of Neuropath. & Exp. Neurol. 17: 12-37, 1958.

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Study of mechanisms involved in infectious diseases.

Principal Investigator: Edwin M. Lerner II

Other Investigators: Leon Sokoloff, Robert R. Williams, Kurt J. Bloch, Joseph J. Bunim, Fletcher P. Williams, Victor H. Haas, Lawrence Kilham, Caspar Hiatt.

Cooperating Units: LPB-NIAMD 10C LAB-NIAMD 117-C LID-NIAID
LBP-DBS LAB-DRS

Man Years (calendar year 1958):
Total: 1
Professional: 1
Other: None

Project Description:

Continued studies of experimental arthritis in rats have produced lesions in 95% of 155 animals injected intravenously with an arthritogenic strain of Streptobacillus moniliformis. The pathogenesis of the acute lesions has been studied in detail, and microscopic changes have been demonstrated as early as 24 hours after infection, although gross changes are usually not visible until the 5th day. Simultaneous bacteriological studies have shown that the microorganism is present in the blood and joint tissues for 48-72 hours after injection, then disappears from both sites, and reappears in the joint at 6 days, suggesting multiplication of the microorganism in the affected joint tissues. Immunological studies of infected rats with acute osteoarthritis have shown a 10-fold increase in plasma opsonins over uninfected animals.

Sera from a large series of infected rats have given elevated titres in the modified sheep cell agglutination test for rheumatoid arthritis, while sera from uninfected rats have had significantly lower or negative titres. This activity has been shown to reside in the euglobulin fraction of rat serum. Sera from infected rats have also reacted positively in the bentonite flocculation test for rheumatoid arthritis, whereas sera from uninfected rats have

generally reacted negatively. Both the modified sheep cell agglutination test and the bentonite flocculation test have been shown to react positively in rats immunized with formalin-killed antigens prepared from S. moniliformis, indicating that these reactions are a response to an antigenic stimulus, independent of active infection or joint inflammation. Rabbits immunized with similar antigens have shown elevated bentonite flocculation test titres. This immunologic response will be pursued, and attempts will be made to characterize the nature of the protein fraction involved, by ultracentrifugation studies, as well as by comparison with other specific and non-specific antigenic stimuli. S. moniliformis has been isolated from the blood of a C. C. patient with high fever and no history of rat-bite. The serum of this patient has shown an elevated sheep cell agglutination titre.

Studies of the filterable "L" forms of the Streptobacillus, as well as of other microorganisms, will be continued. Bacteriological studies of one Clinical Center patient with acute Reiter's disease have employed specialized cultural techniques. A somewhat atypical member of the pleuropneumonia-like organisms has been recovered from the blood, joint fluid, and conjunctiva of this patient, in two separate laboratories. Possible bacteriological studies of other patients are contemplated.

Mice infected with lymphocytic choriomeningitis virus in doses which were otherwise universally fatal were spared by administration of amethopterin. Histological examination of spared animals at intervals ranging from 5 to 57 days revealed greatly modified objective evidence of disease in the majority of these animals. In the acute stage, 82% of the mice showed no disease or extremely modified lesions of LCM. In the chronic stage, 33% showed similarly modified LCM. The remaining mice in both groups showed active lesions of LCM, even as late as 56 days after inoculation. Practically all spared mice gave evidence of multiplication of the virus in the central nervous system. The sparing effect of amethopterin on mice given LCM virus cannot be attributed to suppression of the inflammatory cellular response, nor to eradication or diminution of the virus. The findings suggest that humoral responses of the host are involved, particularly since these spared mice are refractory to subsequent challenge with LCM virus.

Further studies with rabbit myxoma virus have shown that its transforming factor for rabbit fibroma virus is resistant to heat, ether extraction, and to mild enzymatic digestion with desoxyribonuclease and with trypsin. The transforming factor is inactivated by the photodynamic action of toluidine blue, and by ultraviolet irradiation at 2537 Å. It is inactivated by chloroform. The transforming factor particles can be sedimented by ultracentrifugation at 40,000 r.p.m. for one hour, indicating that they are of the same size as the original myxoma virus particles. Electron micrograph studies, although not conclusive, indicate morphological similarity of the above two particles. The transforming factor becomes attached to rabbit kidney cells in tissue culture in 15 minutes, is not destroyed by incubation in tissue cultures for 48 hours at 36° C, and can be added before or after live fibroma virus in order to show its transforming effect. The transforming agent is not immunogenic against myxoma virus in rabbits. All the above diverse lines of evidence point toward the great probability that nucleic acid is involved in, if not synonymous with, the transforming factor.

Part B included: Yes.

Serial No. NIAMD 76

PHS-NIH
Individual Project Report
Calendar Year 1958

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Lerner, Edwin M. II, and Haas, Victor H.: Histopathology of Lymphocytic Choriomeningitis in Mice Spared by Amethopterin. Proc. Soc. Exp. Biol. & Med., 98:395, 1958.

Kilham, L., Lerner, E., Hiatt, C., and Shack, J.: Properties of Myxoma Virus Transforming Agent. Proc. Soc. Exp. Biol. & Med., 98:689, 1958.

Lerner, Edwin M. II, Williams, Robert R., and Jenkins, Jack C.: Sensitized Sheep Cell Hemagglutination Reaction in Rats with Experimental Infection of Bone and Joint. Proc. Soc. Exp. Biol. & Med., 99:249, 1958.

Lerner, Edwin M. II, and Goldoff, Leon: The Pathogenesis of Bone and Joint Infection Produced in Rats by Streptobacillus moniliformis. A.M.A. Arch. Path. In press.

Honors and Awards relating to this project:

Chairman, "Symposium on Pleuropneumonia-like Organisms (PPLO)", Annual Meeting of Society of American Bacteriologists, Chicago, Illinois, April 27-May 1, 1958.

Chairman, Session on Morphology and Classification, New York Academy of Sciences Conference on "Biology of the Pleuropneumonia-like Organisms", New York, N. Y., January 14, 15, 16, 1959.

Designated Discussor of: "Arthritis Caused by Streptobacillus moniliformis and Pleuropneumonia-like Organisms in Small Rodents" and "Skeletal and Articular Involvement in Brucellosis", Conference on "The Comparative Pathology of Arthritis and Rheumatism", American Rheumatism Foundation, Arthritis and Rheumatism Foundation, Nat. Inst. Arthritis & Metabolic Diseases, Bethesda, Md., February 5 and 6, 1959.

...ation on Hematology
...esda, Maryland

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A

Project Title: Pathogenesis of Experimental Arthritis and Pathology of Rheumatism.

Principal Investigator: Leon Sokoloff, M. D.

Other Investigators: None

No parallel research in the Public Health Service.

Man Years (calendar year 1958):

Total:
Professional:
Other:

Project Description:

The findings reported in last year's project have been expanded.

1) Three additional cases of joint involvement in sarcoidosis have been studied and a report submitted for publication.

2) Pathological and clinical studies of rupture of the extensor tendons of the wrist in rheumatoid arthritis have been carried out in 5 patients. A report is being prepared for publication.

3) Two cases of dermatomyositis with vascular lesions have been studied extensively at necropsy and are being reported.

4) Additional instances of little documented lesions include Reiter's syndrome (3), nodular vasculitis (1), Heberden's nodes (1).

5) A systematic study of osteoarthritis of the patella in relation to aging changes in the salivary glands has been initiated. To date, upwards of 20 specimens have been collected and evaluation made.

6) Histopathological studies performed during 1958 in addition to 5 necropsies on arthritic individuals:

Institution	Skin	Muscle	Synovial	Joints dissected
Clinical Center	5	4	0	27
Mt. Alto	8	10	14	2
Others	4	4	4	1

Part B: Honors, Awards and Publications

Sokoloff, L. and Bunim, J. J.: Clinical and pathological studies of joint involvement in sarcoidosis. N.E.J.Med. (in press)

Serial No. NIAMD 78
1. Pathology & Histochemistry
2. Hematology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Regulation of hemopoiesis.

Principal Investigators: Frederick Stohlman, Jr., and George Brecher

Cooperating Units: Medical Department, Brookhaven National Laboratory

Man Years
Total: 5.6
Professional: 1
Other 4.6

Project Description:

Objectives: Study of red cell and white cell turnover with particular emphasis on the determinants of rates of production and the mechanism of action of these regulants. Attention is also devoted to the physiology and clinical identification of erythropoiesis.

Methods employed: Rates of red cell production are determined utilizing Fe^{59} incorporation and apoearance curves. Modification of rates of erythropoiesis is achieved by varying pO_2 concentrations of inspired air, hypertransfusion, blood loss, irradiation and the administration of erythropoietine.

Cellular proliferation in marrow and peripheral blood is being studied by *in vitro* labeling, using tritiated thymidine and radioautographs.

Major findings:

1. The severity of hypoxia governs the rate of production of erythropoietine. The bone marrow cellularity influences the rate of utilization. The balance of these two factors determines the plasma level. E.g. in animals with marrow hypoplasia produced by ionizing radiations the plasma disappearance rate of erythropoietine is slower than in animals with a normal marrow so that for a given level of hypoxia the plasma erythropoietine level is higher in the irradiated animals.

2. Erythropoietine has been demonstrated in the urine of patients with high plasma levels. The chemical nature of the material in plasma and urine appears to be identical. There appears to be a renal threshold for erythropoietine.

3. A dual mechanism for the control of erythropoiesis has been postulated: the hypoxia -- erythropoietine complex and a second mechanism as yet to be defined. The existence of a second mechanism of control was postulated as the result of clinical and experimental observations of alterations in the rate of red cell production in the absence of absolute or relative changes in O_2 delivery or demonstrable erythropoietine production. The working hypothesis that has been advanced for the second mechanism is that an inhibitor of erythropoiesis is produced by senescent red cells and released on the death of the cell.

4. In studies on cellular proliferation it has been demonstrated that an increase in red cell production is achieved through an increase in the number of dividing progenitor cells together with a decrease in the number of normoblasts normally lost by attrition in the marrow rather than by a reduction in intermitotic interval or maturation time.

5. In vitro labeling of marrow and peripheral blood with tritiated thymidine demonstrated the expected high proportion of cells in the stage of DNA synthesis in normal marrow; the presence of occasional mononuclear cells in DNA synthesis in the peripheral blood of normals; an increased number (up to 5%) of such cells in certain infections, particularly of childhood; and a surprisingly low proportion (often less than 5%) of labeled "blast" cells in acute leukemias.

Significance to the program of the Institute: Anemia is a common complication of arthritis and in many instances may be considered to be a metabolic disorder. Understanding of the mechanisms of red cell regulation are not only of basic interest but should eventually result in improved therapy.

Proposed course of project: Studies on the site of production of erythropoietine, its chemical identity and its mechanism of action. Studies directed at elucidating the second mechanism for control of erythropoiesis.

Cooperative work with Chemotherapy Service, General Medicine Branch, NCI, has been started to explore influence of treatment on proliferative capacity of leukemic marrow and blood cells as measured by the thymidine technique.

Part B included Yes

Serial No. NIAMD 78

PHS-NIH
Individual Project Report
Calendar Year 1958

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Stohlman, F. Jr., and Sarnoff, S. J.: Comparison of hemolysis following the insertion of lucite and silastic ball-valves into the circulation system. Proc. 6th Int. Cong. of the Int. Soc. of Hemat., pp 807-812, 1958. Grune and Stratton, N. Y.

Stohlman, F. Jr., and Brecher, G.: The erythropoietic response to hypoxia. Proc. 6th Int. Cong. of the Int. Soc. of Hemat., pp 762-767, 1958. Grune and Stratton, N. Y.

Stohlman, F. Jr.: Observations on hemopoietic effects of radiation -- red cell injury and erythropoietic factor. Proc. 6th Int. Cong. of the Int. Soc. of Hemat., pp 389-395, 1958. Grune and Stratton, N. Y.

Gladner, J. A., Laki, K., and Stohlman, F. Jr.: Labeled DIP-thrombin. Biochim. Biophys. Acta, 27: 218, 1958.

Stohlman, F. Jr.: Observations on the physiology of erythropoietine and its role in the regulation of red cell production. Annals of the New York Academy of Science. In press.

Brecher, G., and Stohlman, F. Jr.: Humoral factors in erythropoiesis. Progress in Hematology, 2nd vol. In press.

Stohlman, F. Jr., and Brecher, G.: Humoral regulation of erythropoiesis V. Relationship of bone marrow activity to plasma erythropoietine level. Proc. Soc. Exp. Biol. and Med. In press.

Brecher, G., Cronkite, E. P., Bond, V. P., and Dutcher, T. F.: Problems of leukocyte transfusion. Acta Hemat. 20: 179-184, 1958.

Brecher, G., Cronkite, E. P., Conard, R. A., and Smith, W. W.: Gastric lesions following single exposures to ionizing radiations. Am. J. Path. 34: 105, 1958.

Hughes, W. L., Bond, V. P., Brecher, G., Cronkite, E. P., Painter, R. B., Quaster, H., and Sherman, F. G.: Cellular proliferation in the mouse as revealed by autoradiography with tritiated thymidine. Proc. Nat. Acad. of Science 44: 476-483, 1958.

Serial No. NIAMD 79
1. Pathology & Histochemistry
2. Hematology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Studies on normal and abnormal hemoglobin.

Principal Investigator: H. A. Itano

Other Investigators: S. J. Singer

Cooperating Units: Department of Chemistry, Yale University (Singer)

Man Years
Total: 2.3
Professional: 1
Other: 1.3

Project Description:

Objectives: To study the physical chemistry, biochemical genetics, and clinical significance of the normal and abnormal hemoglobins.

Methods employed: Moving boundary electrophoresis; spectrophotometry; kinetic and equilibrium measurements with pH meter and spectrophotometer.

Major findings:

Each of the CO-hemoglobins A_2, S_2 and C dissociates in acid into half-units and recombines when neutralized; however, "hybrid" molecules are not detectable when mixtures of two of these forms are recombined (1957 report). Studies during the past year have shown that each molecule dissociates into unlike half-units and that one of the products of dissociation is same for all three forms; therefore exchange of this fragment would not be detectable unless one of the original components of a mixture is labelled. Asymmetric acid-dissociation and hybridization of molecules of hemoglobins A_2, S_2 and C were demonstrated by use of two labelling techniques: (1) mixing the CO-hemoglobin of one inherited form with the ferrihemoglobin of another, and (2) recombining glycine-2-C^{14}-labelled CO-hemoglobin A with unlabelled CO-hemoglobins S and C. Each of the products of acid-dissociation of hemoglobin contains two hemes. Bovine hemoglobin does not dissociate in acid. Canine hemoglobin not only dissociates in acid but also recombines with hemoglobin A to produce two new [illegible]

Significance to the program of the Institute:

The objectives of this program are to inquire into the genetic control of protein structure and synthesis with use of the inherited variants of hemoglobin as experimental tools and to study the relationship of abnormal molecules to inherited anemias. These studies also provide information applicable to the general problems of protein structure and heterogeneity and of the influence of inherited factors to metabolic disease. Each of the hemoglobins is a symmetrical molecule. A heterozygous red cell produces different symmetrical molecules but does not produce any mixed molecules; therefore a mechanism of synthesis whereby two like halves are synthesized in close proximity is suggested. These studies have been extended to other species in order to determine the effects of larger genetic changes on molecular properties.

Proposed course of project: Further attempts will be made to produce pairs of unlike peptides by carrying out the dissociation of hemoglobin under more drastic conditions. The structural relationship of hybrid molecules to the intermediate compounds of hemoglobin reactions will be investigated. In this connection a search for other methods of labelling the hemoglobin molecule will be made.

Part B included Yes

Serial No. NIAMD 19

PHS-NIH
Individual Project Report
Calendar Year 1958

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Itano, H. A., and Singer, S. J.: On dissociation and recombination of human adult hemoglobins A, S, and C. Proc. Natl. Acad. Sci. 44: 528-529, 1958.

Itano, H. A., and Robinson, E.: Electrophoretic separation of intermediate compounds in the reactions of ferrihemoglobin. Biochimica et Biophysica Acta 29: 545-555, 1958.

Itano, H. A.: Electrophoretic analyses of abnormal human hemoglobins. In "Conference on Hemoglobin", Publication 557 of National Academy of Sciences -- National Research Council. Washington, D. C., pp 14-15, 1958.

Itano, H. A.: Genetic and physical factors in the heterogeneity of hemoglobin. In Proceedings of the Istanbul Symposium on Abnormal Hemoglobins. Blackwell, London, in press. 1958.

Itano, H. A.: Interpretation of data on hemoglobin heterogeneity. In Proceedings of the Istanbul Symposium on Abnormal Hemoglobins. Blackwell, London, in press. 1958.

Serial No. NIAMD 80
1. Pathology & Histochemistry
2. Hematology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Sulfhydryl (Mercapto) Groups of Hemoglobin
Studies on the nature of the mercapto-mercapto interaction.

Principal Investigator: Makio Murayama

Other Investigators: none

Cooperating Units: none

Man Years Came on duty 1st of October
Total:
Professional:
Other:

Project Description:

Proposed course of project: Sulfhydryl groups of hemoglobins will be studied by means of heavy metal ion binding using the platinum wire electrode as an indicator electrode. The data thus obtained will be analyzed to find out to what extent the sulfhydryl groups interact. It seems that the sulfhydryl-sulfhydryl interaction is extremely important for the understanding of heme-heme interaction accompanying oxygenation.

It is also proposed that a specific chemical difference between the normal adult human hemoglobin and an abnormal hemoglobin I will be studied. This investigation will be carried out by a method known as "finger printing" technique: a tryptic digest of the protein will be made; the hydrolysate is then subjected to electrophoresis on filter paper and then followed by chromatography to separate the peptides.

Part B included No

Serial No. NIAMD 61
1. Pathology & Histochemistry
2. Hematology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Studies of dietary factors and embryonic tissues as affecting rat leprosy and experimental amyloidosis.

Principal Investigator: George L. Fite

Man Years
Total: 0.5
Professional: 0.5
Other: -

Project Description:

Objectives:

1. To investigate the effect of vitamin E deficiency on rat leprosy, following the lead of Bergel and Mason in their work on human leprosy, in which they reported enhancement of the infection in rats fed E-deficient diet rich in cod liver oil. This work, if true, may be of great significance, and appears of readily susceptible analysis using rat leprosy.

2. To explore further the unreported work of H. S. N. Greene in transplantation of lepromas to brain tissue. It is planned to explore this field widely: by implantation of infected embryonic tissues into non-susceptible animals, not only into brain, but testis and other organs.

3. To examine the infectivity of M. Leprae Murium growing in the ascites tumor of the mouse.

4. To analyse experimental amyloidosis in animals using several dietary procedures and rat leprosy as the readily available causative agent.

Methods employed: Animal experimentation.

Major findings: None

Proposed course of project: Duke terminated at NIH July 1, 1958.

Part B included No

Serial No. NIAMD 02
1. Pathology & Histochemistry
2. Hematology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Pathogenesis of Experimental Arthritis and Pathology of Rheumatism.

Principal Investigator: Leon Sokoloff, M. D.

Other Investigator: Emanuel Silverstein, M. D.

Cooperating Units: NIAMD, LNE, Dr. Mickelsen 2
RLA, Dr. Jay
NIAMD, LPH, Dr. Lerner 76
NCI, Dr. Deringer
NIDR, Dr. Rizzo
NIAMD, Dr. Blumberg 117-C

Man Years
Total: 3.0
Professional: 1.5
Other: 1.5

Project Description:

Objectives: Investigation of factors influencing development of degenerative joint disease in small laboratory animals; production of inflammatory arthritis.

Methods employed: The role of genetic factors in osteoarthritis is being studied by anatomical F1 and F2 hybrids, backcrosses and reciprocals of certain mice are being made. A survey of several biochemical characteristics is to be carried out.

Necrotizing arteritis in an inbred strain of mice is being studied anatomically and chemically.

Major findings: The experiments described in the last project report have been completed and are now undergoing statistical analysis. The osteoarthritis of mice is clearly recessive. Preliminary survey of mice long surviving total body irradiation (Dr. A. C. Upton, Oak Ridge National Laboratory) discloses no potentiation of osteoarthritis. The infection of bone and joint produced by Streptobacillus moniliformis has been studied in collaboration with Dr. Lerner. Although it results in positive serological tests for rheumatoid arthritis, the lesion has its inception in the bone.

Proposed course of the project: The genetic project is well under way and will be completed in two years. It is anticipated that the number of genes involved will be determined from this. Development of a large colony of BI/HeN mice is underway for investigation of the pathogenesis of the arteritis that resembles in several respects the vascular lesions of human rheumatic diseases. This project will also require at least two years. The potentialities of establishing biochemical peculiarities of the various mouse strains; further systematic study of total body irradiation on osteoarthritis; and sialadenectomy on osteoarthritis in rats can better be evaluated after further work is done.

Part B included Yes

Serial No. NIAMD 82

PHS-NIH
Individual Project Report
Calendar Year 1958

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Sokoloff, L., and Habermann, R. T.: Idiopathic necrosis of bone in small laboratory animals. AMA Arch. Path. 65: 323-330, 1958.

Silverstein, E., and Sokoloff, L.: Natural history of degenerative joint disease in small laboratory animals. 5. Osteoarthritis in guinea pigs. Arth. Rheum. 1: 82-86, 1958.

Sokoloff, L.: Joint diseases of laboratory animals. J. Nat. Canc. Inst. 20: 965-978, 1958.

Sokoloff, L.: Designated discussion: The role of mycobacteria in allergic manifestations. Henry Ford Symposium. In press.

Lerner, E. M., and Sokoloff, L.: The pathogenesis of bone and joint infection produced in rats by Streptobacillus moniliformis. AMA Arch. Path. In press.

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

ANNUAL PROJECT REPORT

CALENDAR YEAR 1958

Summary Sheet

LABORATORY OF PHARMACOLOGY AND TOXICOLOGY

Estimated Obligations for FY 1959

Total - $671,911
Direct - $409,200
Reimbursement - $262,711

Project Numbers 83 thru 97 Included

FWB-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Mechanisms and therapy of traumatic shock.

Principal Investigator(s): Drs. K. Merideth, M. Becerra, N. A. Mefalides, R. C. Millican and S. M. Rosenthal

Other Investigator(s): A group of Peruvian surgeons

Cooperating Units: Hospitals Loayza, del Nino, and dos de Mayo

Man Years (calendar year 1958):
Total: 8
Professional: 7
Other: 1

Project Description:

Objectives:

(1) Clinical evaluation in burn shock of plasma and of large volumes of sodium solutions.

(2) Adrenocortical function in burn trauma.

Methods Employed:

(1) The clinical study in Lima, Peru involves comparison of saline solutions alone, chiefly by mouth, with plasma or with plasma plus saline solutions. Extensive corrolary laboratory studies in hemodynamics, bacteriology, and renal function are carried out.

(2) The renal excretion of steroids after a standard infusion of 25 mg. of ACTH has been taken as a measure of adrenal cortex activity.

Major Findings:

(1) In adults more than 80 cases of extensive burns have been treated with saline solutions alone, with no deaths from shock. Eleven of these had burns greater than 50 per cent of the body and three greater than 75 per cent. In the control cases treated alternately with plasma plus glucose and water 12 per cent died of shock. The delayed deaths were similar in both groups. In 73 burned children treated with saline the acute mortality from shock was 1) per cent and the delayed mortality 47 per cent. In 91 children treated with

Part B included [x] Yes [ ] No.

saline plus plasma the acute mortality was 9 per cent and the delayed mortality 35 per cent. Some evidence of an added beneficial effect from plasma was thus obtained in children.

(2) The infusion of ACTH on the 3rd and 4th post burn days in patients with 10-25% body surface burns produced a 9.0 and 21.9 mg. average rise respectively in urinary excretion of 17-hydroxysteroids compared with a control group of patients. In patients with 26-50% burns, however, average 17-hydroxycorticosteroids excretions were 5.6 mg. higher on the 3rd post burn day and 1.2 mg. lower on the 4th post burn day in the ACTH group than in the control group on the days of ACTH infusion. Thus it is demonstrated that there was maximal stimulation of the adrenal cortex in the more extensive burns. Ketosteroid excretion was not significantly affected by ACTH.

Proposed Course of Project:

(1) Continued study of basic mechanisms that produce shock and the effect of therapeutic measures.

(2) Continued evaluation of saline and plasma therapy, along with certain plasma substitutes, as human serum albumin and dextran.

Part B: Honors, Awards, and Publications

Millican, R. C.

Fluid, electrolyte and plasma protein disturbances in shock. Symposium on Biochemical Response to Physical Injury, sponsored by C. I. O. M. S. of UNESCO at Semmering, Austria, September 1958. (To be published in book form.)

Rosenthal, S. M.

Some aspects of the therapy of injury. C. I. O. M. S. Symposium, Semmering, Austria, September 1958. (To be published in book form.)

1. Laboratory of Pharmacology and Toxicology
2. Section on Pharmacology
3. Bethesda, Md. and Lima, Peru

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: The role of gamma globulins in resistance to infections from organisms of low virulence.

Principal Investigator(s): Drs. R. C. Millican, N. A. Kefalides, K. Markley, and S. M. Rosenthal

Other Investigator(s): J. D. Rust

Cooperating Units: Hospitals Loayza, del Nino, and dos de Mayo

Man Years (calendar year 1958):
Total: 5
Professional: 4
Other: 1

Project Description:

Objectives:

(1) The effectiveness and mechanisms of action of certain drugs in shock.

(2) Clinical evaluation in burn shock of large volumes of saline solutions.

(3) To obtain more patent antiserum by immunization of animals with Pseudomonas and Staphylococci.

(4) Evaluation of gamma globulin and antibiotics in experimental infections and in clinical infections following burns (Peru Project).

Methods Employed:

(1) The production of fatal infections with organisms of low virulence in animals made susceptible by burn or tourniquet trauma, by cortisone or by injecting the organisms in mucin.

(2) Assay of protective titres of various sera against these infections

Part B included [x] Yes [ ] No

(3) Hemagglutinin, complement fixation and other immunological tests on these sera.

(4) (Peru Project) Extensive bacteriological and immunity studies on burned patients.

Major Findings:

Experimental Study.

(1) A rabbit Pseudomonas immune serum was prepared by intravenous injection of live Pseudomonas aeruginosa organism. This antiserum afforded over a thousand fold greater protection against fatal mouse infection than human gamma globulin and was effective 6 hours after the experimental infection. Antiserum protected against infections from 5 different strains of Pseudomonas aeruginosa and 4 different organisms isolated from patients with Pseudomonas aeruginosa septicemias.

(2) Serological studies were made of human gamma globulin, Pseudomonas antiserum and the plasmas from different animal species that were effective against experimental Pseudomonas infection. Agglutination (qualitative and quantitative), complement fixation and hemagglutination reactions showed a general correlation with in vivo protective titers.

Sera from germ-free and conventional rats were equally protective against Pseudomonas and Staphylococcal infections. However, the germ-free serum showed titres with the hemagglutination reaction against Pseudomonas that were very low or absent.

(4) Rabbit Staphylococcal immune serum was prepared by intravenous injection of live organisms of Smith strain of Staph. aureus. The protection of this immune serum against fatal mouse infection was several thousand fold greater than of normal rabbit serum. In contrast to the complete protection by Pseudomonas immune serum against all strains of Ps. aeruginosa studied, the Staphylococcal immune serum protected against infections from only 2 strains of Staph. aureus of a total of 6 strains examined.

Peru Project: The prophylactic effect of gamma globulin in preventing systemic infections in burns was tested by giving three doses of 1 ml. per kilo during the first 10 days post burn to children and 50 ml. to adults. A high incidence of Pseudomonas and Staphylococcus septicemia has been encountered in the cases in Peru and antibiotic therapy has proven ineffective. While the study will require several years for a definitive answer, results for the first year show 12 cases of septicemia in 42 controls and 2 cases in 29 gamma globulin treated cases. The 60 cases received similar antibiotic therapy as the controls.

Accessory bacteriological studies showed that the burned area, the pharynx and the gastrointestinal tract rapidly become contaminated with these organisms, and that antibiotics did not prevent the spread of infection. Antibiotics favored the emergence of resistant strains.

Proposed Course of the Project:

(1) Continued study of the role of infection and metabolic disturbances in late deaths following burns in experimental animals and man.

(2) Further evaluation of normal gamma globulins and of globulins from immunized animals in experimental and clinical infections. An immune rabbit serum against Pseudomonas is being prepared by Lederle Laboratories for this purpose.

(3) Immunological studies on the nature of the protective action against organisms of low virulence.

Part B. Honors, Awards, and Publications

Gamma globulin against infections with organisms of low virulence. Symposium, Dallas, Texas (Ross Laboratories), 1958. (To be published in book f

and Toxicology
2. Section on Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1958

Part I.

Project Title: Estimation, metabolism and function of spermine, spermidine and related amines, and of the basic proteins.

Principal Investigator(s): Drs. S. M. Rosenthal, C. W. Tabor, and D. Dubin

Other Investigator(s): Dr. H. Tabor

Cooperating Units: Section on Biochemical Pharmacology, LPT NIAMD 91

Man Years (calendar year 1958):
Total: 2-2/3
Professional: 1-2/3
Other: 1

Project Description:

Objectives: The importance of these amines and of protamines and histones is shown by their wide distribution in nature. Studies are conducted to elucidate the metabolism and function of these substances, and their relation to desease.

Methods Employed:

(1) The amines are studied by isotopic labeling ($C^{14}N^{15}$) and by special chromatographic techniques.

(2) Methods have been recently devised for the estimation of protamines and histones in tissues.

Major Findings:

(1) Biosynthesis of spermidine (see report from Biochemical Pharmacology Section).

(2) E. coli cells, when grown at pH 8 on a basal medium, were shown to contain acetyl derivatives of putrescine and spermidine. Acetyl putrescine approximated 1 micromole per gram compared to 5 micromoles of putrescine. Acetyl spermidine approximated 2 micromoles per gram compared to around 1.8 micromoles of spermidine. Normally E. coli does not contain spermine, but when spermine is added to the medium both spermine and mono and diacetyl spermine can be demonstrated by paper and columnar chromatography in extracts of the cells. This demonstration of acetylation of aliphatic amines is of interest because little is known of this type of reaction in bacteria.

Part II included [x] Yes [ ] No

(3) In collaboration with Dr. Ames of the Biochemical Pharmacology Section, the amine content of several viruses has been studied. With T_4 phage the amine content of the virus reflects that of the host cell, and both can be influenced by the amine content of the culture medium. (See also Biochemical Pharmacology Section.)

(4) Satisfactory chromatographic methods for estimation of protamines and histones have previously been lacking. It was found that histones could be eluted from IRC-Na (XE-64) resins on the alkaline side by low concentrations of sodium lauryl sulfate. It was also found that protamines could be eluted from the hydrogen resin by dilute acids. Three components were observed with histone preparations, while a single peak was obtained with three protamine preparations (salmine and clupeine). The procedures have been adapted to the rapid estimation of these basic proteins in crude tissue extracts.

Proposed Course of Project:

(1) To complete our knowledge of the biosynthesis and fate of these polyamines, and to relate these findings to their physiological and pharmacological effects.

(2) To elucidate the bound state in which they exist in cells.

(3) To further study the renal toxicity, bactericidal and spermatocidal effects in their relation to physiological function and possible role in disease.

(4) To study the distribution of the basic proteins in various tissues and cell preparations under various physiological conditions.

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

(1) Ames, B. N., Dubin, D. T., and Rosenthal, S. M.: Presence of polyamines in certain bacterial viruses. Science 127: 814-816, 1958.

(2) Tabor, H., Rosenthal, S. M., and Tabor, C. W.: The biosynthesis of spermidine and spermine from putrescine and methionine. J. Biol. Chem. 233: 907-914, 1958.

Serial No. 44
1. Laboratory of Pharmacology and Toxicology
2. Section on Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Physicalchemical and metabolic factors relating the action of physiological and pharmacological agents in excitable cells such as nerve and muscle.

Principal Investigator(s): A. M. Shanes

Other Investigator(s): N. L. Gershfield and C. Paul Bianchi (PHS Fellow and Visiting Scientst)

Cooperating Units: Dr. S. Winegrad, Heart Institute

Man Years (calendar year 1958):
Total: 4
Professional: 3
Other: 1

Project Description:

Objectives: The interrelationships that have been established between monovalent ion movement and bioelectrical properties through studies of the action of metabolic inhibitors and substrates, drugs, and ions on nerve have been extended to include muscle contractility and surface films and the possible role of the multivalent ion calcium. Our present interest is to determine (a) to what degree the details of calcium distribution and transport in connective tissue and muscle (skeletal and cardiac) can be elucidated and correlated with changes in the ionic milieu and with the strength of muscular contraction and (b) to what extent surface films of simple molecules and of cellular extracts, and more rigid thin membranes, will continue to exhibit the same specificity and sensitivity to pharmacological and physiological agents as excitable cells.

Methods Employed:

(1) The rate of entry and exit of the radioisotope of calcium, Ca^{45}, in skeletal muscle and in the tendon of Achilles (frog) are measured in media modified with respect to the ions and also compared with the strength of muscular contraction.

Part B Included [x] Yes [ ] No

(2) Surface-films of stearate and myelin extracts are subjected to drugs and ions well characterized physiologically and pharmacologically; the action of these agents is evaluated by the manner in which they alter the spreading pressure and molecular area of the surface-films. The physical properties of the films and of the interacting molecules (e.g., pK, surface activity, size, and shape) are related to the interactions observed.

(3) Semi-rigid films of considerable thinness (e.g., 100 Å), composed in part of molecules in (2), are constructed so that the augmented diffusion potentials and the electrical resistance to which they give rise can be followed as affected by physiological and pharmacological agents applied to either of the two sides.

(4) The entry and exit of the radioisotopes of sodium and chloride as well as of the non-radioactive ions in sciatic nerves of the toad have been followed as affected by alterations of the ionic milieu. The effectiveness of veratrine and cocaine in modifying ionic movement has also been observed at different levels of high hydrostatic pressure.

Major Findings:

(1) The relation of calcium entry to muscular contraction. The rate of entry of calcium into unstimulated muscle cells, is the same as in unstimulated nerve fibers, but thirty times greater than in nerve during an impulse. The passive uptake rate is proportional to extracellular concentration whereas the entry during stimulation is unaffected by concentration. When the strength of a twitch is increased by replacing the chloride of the medium with nitrate, calcium uptake is increased in the same proportion. The shortening of skeletal muscle by potassium also is accompanied by a brief enhanced entry of calcium. These findings suggest (a) that the entry of calcium at a sufficiently high rate initiates contraction and (b) that the amount entering may be one factor governing the strength of contraction.

(2) Distribution and outward transport of calcium in skeletal muscle and tendon. The Ca^{45} taken up by tendon is exactly double that to be expected from simple equilibration of its water content with "hot" solutions. An analysis of the kinetics of the emergence of Ca^{45} from muscle and tendon previously equilibrated in Ca^{45} media leads to the conclusion that in muscle calcium is present in the interfibrillar water, on superficial sites present in the connective tissue and on the muscle fibers, and within the muscle fibers. The proportions of exchangeable calcium in the three superficial regions of muscle are approximately the same and they total about half that inside the fibers. The calcium located on the surface sites of the muscle fibers is distinguishable from that on connective tissue by virtue of its being bound much more tightly when NO_3^- replaces Cl^-; consequently it is not released from the sites in non-radioactive solution. These findings provide a basis for the augmented uptake of calcium by muscles stimulated in nitrate solutions.

(3) The interactions of ions and drugs with surface-films of stearate. Excitatory alkaloids such as veratridine and cevadine, present in veratrine, as well as local anesthetics such as cocaine interact with these films at the same concentrations as they act pharmacologically on living excitable cells. The only effect of the latter drugs is to enter the film and thereby to increase

the spreading force. The interactions involved are weak and are counteracted by compression of the film and by low calcium concentrations. The former, on the other hand, interact much more strongly to increase the spreading force of the film, and also to form a soluble salt complex with the stearate. The instability so induced can be counteracted by low concentrations of calcium.

Proposed Course of Projects: Our calcium studies provide a basis for exploring deviations in the distribution and metabolism of calcium that may occur in pathological states including arthritis. It now appears feasible, too, to characterize normal and abnormal tissues by changes in the calcium association coefficients of individual tissues. The enhancement of calcium turnover by muscular activity has important implications for the atrophy of muscle with disuse, and for derangement of calcium metabolism during extended muscular inactivity.

The findings to date on surface films suggest the possibility of their use for bioassay purposes as well as for a means of exploring interactions that closely parallel and hence may underlie a number of drug and ion effects in living nerve and muscle. It is possible to describe the alignment of the molecules in the film as well as the nature of certain of the interactions, which renders this approach an important and sensitive supplement to studies restricted to measurement of enzymatic processes and enzymatic inhibition.

Now that techniques for measuring different forms of bound calcium in connective tissue and muscle have been worked out, it is desirable to characterize the binding sites by studying the effect of pH, competing ions, and metabolic inhibition and substrates on binding-concentration curves. The relation of the strength of skeletal muscle contraction to the entry of calcium and other multivalent ions, as affected by other ions and drugs, remains to be explored. Available literature suggests the desirability of examining heart and smooth muscle from this standpoint, the former of which is already under study by Dr. Saul Winegrad, who hopes to join our group.

The study of surface-films will, as facilities permit, extend in three directions:

(1) Studies with other drugs and ions, the physicochemical effects of which in excitable cells are well characterized, to determine how general is the specificity and parallelism of film interactions.

(2) Studies with semi-rigid films that permit measurements of resistance, electrical potential, and ion movement to verify that the physicochemical changes observed in cells can be duplicated in simple models; preliminary experiments with veratrine and cocaine have been promising already.

(3) Studies with films composed of molecules of greater complexity, e.g., the individual components of cellular lipids and purified extracts from tissues with membranes exhibiting different electrochemical properties (e.g., chemically excitable membranes such as at junctions as contrasted with electrically excitable membranes).

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

(1) Shanes, A. M.: Electrochemical aspects of physiological and pharmacological action in excitable cells. I. The resting cell and its alteration by extrinsic factors. Pharmacol. Rev. 10: 59-164, 1958.

(2) Shanes, A. M.: Electrochemical aspects of physiological and pharmacological action in excitable cells. II. The action potential and excitation. Pharmacol. Rev. 10: 165-273, 1958.

(3) Gershfeld, N. L. and Shanes, A. M.: The influence of high hydrostatic pressure on cocaine and veratrine action in a vertebrate nerve. J. Gen. Physiol., Jan. 1959. (Accepted for publication.)

(4) Bianchi, C. P. and Shanes, A. M.: Calcium influx in skeletal muscle at rest, during activity, and during potassium contracture. J. Gen. Physiol., March, 1959. (Accepted for publication.)

(5) Shanes, A. M., Freygang, W. H., Grundfest, H., and Amatniek, E.: Anesthetic and calcium action in the voltage-clamped squid giant axon. J. Gen. Physiol., March, 1959. (Accepted for publication.)

(6) Shanes, A. M.: Some aspects of myelin in nerve trunks. In "Progress in Neurobiology", (ed): Korey, S. R. and Nurnberger, J. I. N. Y. Hoeber, in press.

Honors relating to this project:

(1) Dr. Shanes has been invited to serve for one year as Liaison Biophysicist in the London Branch of the Office of Naval Research. In this capacity he will visit foreign laboratories to evaluate the current status of research and of scientists in his field in foreign countries and expedite the interchange of information and scientists between the U. S. and abroad.

Serial No. 87
1. Laboratory of Pharmacology and Toxicology
2. Section on Pharmacology
3. Bethesda, Md.

PHS - NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: The chemotherapy of mouse leprosy

Principal Investigator: Dr. Y. T. Chang

Other Investigator(s): None

Cooperating Units: The American Leprosy Foundation, Leonard Wood Memorial

Man Years (calendar year 1958):
Total: 2
Professional: 1
Other: 1

Project Description:

Objectives: The evaluation of therapeutic effectiveness of drugs in mouse leprosy. The tissue culture of intracellular parasites.

Methods Employed: Intraperitoneal infection of mice with M. lepraemurium. Tissue culture of monocytes by a test tube method specially adapted in this laboratory.

Major Findings: Kanamycin showed strong suppressive activity in mouse leprosy; 4,4'-diaminodiphenyl sulfide, 4-amine-4'-ethylenediaminodiphenyl sulfone, SU 3068 (a thiazoline compound), 3 isoniazid derivatives (n-heptyl, 4-methyl-2-pentyl and 2:6-dimethyl-4-heptyl), only weak activity; amodiaquin, Y 9525 (a diphenylthiourea derivative), and three Indian plant drugs (pomegranate, myrobalan and lead wort), no activity.

Studies on the development of DDS-resistance in mouse leprosy are completed. Leprous mice were treated with DDS continuously through 12 successive transfers in a total of 62 months in one experiment, and 8 successive transfers in 45 months in the other. Measurable differences in lesions between the untreated and treated animals were observed at the end of both experiments, although the effectiveness of the drug was decreased.

Part B included [x] Yes [ ] No

Marked improvement in the cultivation of Mycobacterium leprae murium in tissue culture of monocytes was obtained by using a medium containing unfiltered horse serum supplemented with freshly prepared spleen homogenates from young mice. Monocytes grew well for more than 12 weeks in such medium, although the cell population decreased gradually during cultivation. Cultures infected in vitro with bacillary suspension showed elongation of bacilli as early as 5 days after infection and became more marked afterwards. Elongation was also observed in cultures using pure filtered horse serum or diluted unfiltered horse serum. However, the cell population maintained much better with the supplement of spleen homogenates. In a total of about 50 trials, definite elongation of bacilli was observed in all with only one failure. The number of bacilli increased steadily. Heavily infected cells could be seen under low power objective in 3 or 4 weeks cultivation. The total number of bacilli remained in the doubled at the end of 5 weeks in one experiment, and increased 7 fold at the end of 7 weeks in the other, although the cell population was only about 50% of the original at this time in both experiments. Cultures obtained from cells infected in vivo (i.e., in mice which had been infected previously for various length of time) showed more marked growth than those infected in vitro. Cultures infected with killed bacteria or treated with isoniazid, 1 or 5γper ml. showed no growth. Streptomycin 1, 5 or 50γ per ml. was inactive.

Monocytes infected with tubercle bacillus BCG or H37Rv grew well in this system. Good growth of intracellular bacilli was obtained by adding streptomycin to the medium in order to check the extracellular growth of bacilli. Once there was growth of bacilli in the medium the cell population diminished rapidly.

Proposed Course of Project: The leprosy studies are carried out in cooperation with the American Leprosy Foundation (Dr. Chang is on a Fellowship from them). The results are applied to their clinical evaluation studies.

Continuation of evaluation of drugs in mouse leprosy, using both long-term and short-term techniques. Continuation in the study of tissue culture of Mycobacterium leprae murium and Mycobacterium tuberculosis.

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

(1) Chang, Y. T.: Chemotherapy of murine leprosy. VII. The effect of cycloserine (Seromycin) on mouse leprosy. Internat. J. Leprosy, 25: 257-262, 1957.

(2) Chang, Y. T.: Evolution of murine leprosy. Amer. Rev. Tuber. & Pulm. Dis. In press.

(3) Chang, Y. T.: Effects of kanamycin, streptovaricin, paromomycin, novobiocin and vancocetin on murine leprosy. Amer. Rev. Tuber. & Pulm. Dis. In press.

Serial No. 22
1. Laboratory of Pharmacology and Toxicology
2. Section on Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Toxicologic studies of iodates

Principal Investigator(s): Dr. S. H. Webster

Other Investigator(s): Mr. E. F. Stohlman

Cooperating Units: Dr. Benjamin Highman, NIAMD Pathology 74
Dr. Ralph Gunkel, NINDB Retinal Examinations

Man Years (calendar year 1958):
Total: 2-2/3
Professional: 1-2/3
Other: 1

Project Description:

Objectives: The toxicology of iodates as a basis for use in iodised salt.

Methods Employed: Determination of chronic toxicity of KIO_3 in dogs, by oral administration at three levels for one year; study of distribution and excretion of KIO_3, after various routes of administration to rodents; investigation of analytical methods for determination of iodate and iodide in biological materials; study of reduction of toxic effects of iodates by protective agents.

Major Findings: The dogs on the low dosage level of 30 mg/kg/day of KIO_3 received about 1000 times as much iodate per kilogram as a 70 kg human being would consume daily in 15 g of salt containing 0.01% KIO_3. This group of animals was not adversely affected with respect to maintenance of weight, physical condition, appetite, or food consumption. No significant changes in urine and no clinical evidence of impairment of vision were found. A slight decrease in red cell count and slight reticulocytosis were noted in some of the dogs toward the end of the year. No increase occurred in the methemoglobin content of the blood. The most constant histopathologic finding was a mild inflammation of some portions of the gastrointestinal tract.

A comprehensive report concerning the toxicology of potassium and sodium iodates, with particular reference to the proposed use of these substances in iodating salt for culinary use, has been prepared and sent to the

Part B included [ ] Yes [ ] No

Food and Drug Administration and to the Nutrition Section, Pan American Sanitary Bureau for use by these organizations.

A study was made of the distribution of iodate and iodide following administration of these substances to rats, guinea pigs and rabbits. The apparent absence of iodate and/or iodide in important organs, as liver and kidneys, led to an investigation of existing analytical methods. It was found that the sensitivity of the tests for iodate or iodide, which depend on the liberation of iodine and subsequent action on starch to form a blue substance, may be greatly reduced by interfering substances present in tissues. The nature of these interferences has been investigated and methods of detection have been devised for use with urine, plasma, gastrointestinal contents and certain organs.

In vivo and in vitro studies with iodate have shown that conditions are favorable in the stomach and duodenum for the reduction of small amounts of iodate to the less toxic iodide. With such protective mechanisms available, it is highly unlikely that any toxic effect would be produced by ingestion of cooking salt containing potassium iodate in amounts up to 0.01%.

Proposed Course of Project: On the basis of the findings made in this study, potassium iodate, KIO_3, appears to be a safe substitute for the unstable potassium iodide, KI, at present used to iodize cooking salt and salt-feed mixtures for cattle. A trial period for use of KIO_3 in salt, in amounts not to exceed 0.01% or its equivalent as $NaIO_3$, with a subsequent survey to evaluate the results, is recommended.

Further work on the absorption and fate of iodate, using improved analytical methods, is expected to clarify some of the unexplained phenomena, such as the rapid excretion of iodate followed by slow excretion of iodide after oral or intraperitoneal injection of iodate, or the apparent absence of iodide or iodate in bile. The binding of iodide and iodate by tissues will also be investigated.

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

(1) Webster, S. H., Rice, M. E., Highman, B., and Stohlman, E. F.: The toxicology of potassium and sodium iodates. II. Subacute toxicity of potassium iodate in mice and guinea pigs. Toxicol. & Applied Pharmacol. Accepted for publication. To appear in January, 1959

Serial No. 89
1. Laboratory of Pharmacology and Toxicology
2. Section on Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Fatty changes in mice induced by short-time fasting

Principal Investigator(s): Dr. S. H. Webster

Other Investigator(s): Mr. E. F. Stohlman

Cooperating Units: Dr. B. Highman, NIAMD 74

Man Years (calendar year 1958):
Total: 2
Professional: 1-1/3
Other: 2/3

Project Description:

Objectives: Determination of the duration of fasting necessary to produce observable fatty changes in such organs as liver, heart, kidneys and adrenals of mice and the time required for reversal of such changes.

Method Employed: Determination of total fat content and gross and microscopic examination of above organs before, during, and after fasting.

Proposed Course of Project: This study should give a better insight into what changes, fatty or otherwise, are likely to occur in the organs of mice due to withdrawal of food for periods of 4 to 48 hours. Such information should be of great value in toxicological studies where fasted mice are used. It is sometimes uncertain at autopsy whether fatty changes are caused by voluntary fasting, involuntary fasting, or action of an administered drug. This is of particular importance in oral toxicity studies.

This work can be carried on simultaneously with the remainder of the iodate study. Some work has already been done. Pathologic examinations will be carried out by Dr. Benjamin Highman.

Part B included [ ] Yes [x] No

Serial No. 90
1. Laboratory of Pharmacology
2. Section on Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
Calendar Year 1958

I. Scientific Activities:

1) A voluminous paper "The ammobasic saturated aliphatic acids and their esters, their toxicity and potential dangers" was prepared and is now being copied.

2) A second paper "The ammobasic unsaturated aliphatic acids and their esters, their toxicity and potential dangers is in preparation.

3) The literature on clinical cases of poisoning and of side-actions of drugs was followed. This entails the study of approximately 150 publications per month.

II. Consultant Activities:

1) Numerous letters in answer to requests for information on toxicological subjects were written and similar telephone calls were answered.

2) Acted as expert witness for the Food and Drug Administration on four, and for the Armed Forces on the occassion.

III. Other Activities:

1) Attended two sessions of the Advisory Commission on Environmental Hygiene to the Armed Forces.

2) Attended four meetings of the Interdepartmental Committee on Pest Control.

3) Participated in a conference on poisoning at Walter Reed Hospital.

4) Attended the President's Conference on Occupational Safety.

5) Directed the activities of the Pest Office Advisory Committee.

6) Directed and participated in the development of standards on maximum acceptable concentration of chlorinated hydrocarbons for the Committee Z37 of the American Standards Association.

7) Participated in the activities of the PHS Chemical Products Labeling Committee.

Serial No. NIAMD- 9L
1. Pharmacology & Toxicology
2. Biochemical Pharmacology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Putrescine, Spermidine, and Related Polyamines

Principal Investigators: Sanford M. Rosenthal, Celia White Tabor, and Herbert Tabor

Other Investigators: Bruce Ames and Donald Dubin

Cooperating Units: (in collaboration with Section on Pharmacology, LPT NIAMD) 8S

Man Years (calendar year 1958):
Total: 3
Professional: 2-1/2
Other: 1/2

Project Description:

Objectives: To study biosynthesis of these polyamines, and their role in bacterial, virus, and mammalian metabolism.

Major Findings:

1. Enzymes have been purified from E. coli which carry out the following reactions:

(1) ATP + L-methionine $\xrightarrow{Mg^{++}}$ S-adenosyl-L-methionine

(2) S-Adenosyl-L-methionine $\xrightarrow{Mg^{++}}$ CO_2 + S-adenosyl (5')-3-methylmercaptopropylamine

(3) S-Adenosyl (5')-3-methylmercaptopropylamine + putrescine → spermidine + [thiomethyladenosine]

Enzyme I has been purified about 90 fold. This reaction requires Mg and ATP and has a pH optimum of 8. Enzyme II has been purified 25 fold. It requires Mg, has a pH optimum of about 7, and is inhibited by CN. The product S-adenosyl-methylmercaptopropylamine has been isolated and serves as substrate for step III. Enzyme III has been purified 30 fold. No cofactors have been found and the pH optimum is around 8.

Part B included [X] Yes [ ] No

2. Putrescine and spermidine have been identified as basic substances in bacteriophage T_4, accounting for about 45 per cent of the phosphate groups in the DNA.

Significance to NIAMD Research: These polyamines are widely distributed in animal and microbiological material, although their function is not yet known. The phage studies indicate a possible role in nucleic acid structure or metabolism. The enzymatic studies are of interest not only in showing how these amines are made but also in demonstrating another metabolic role for adenosylmethionine.

Proposed Course of Project:

1. Further purification and characterization of each enzyme.

2. Further studies on the possible metabolic role for these amines in animal and microbiological tissues.

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Individual Project Report
Calendar Year 1958

Part B: Publications

Publications other than abstracts from this project:

(1) Ames, B. N., Dubin, D. T., and Rosenthal, S. M.: Presence of polyamines in certain bacterial viruses. Science 127: 814-816, 1958.

(2) Tabor, H., Rosenthal, S. M., and Tabor, C. W.: The biosynthesis of spermidine and spermine from putrescine and methionine. J. Biol. Chem. 233: 907-914, 1958.

Serial No. NIAMD- 92
1. Pharmacology & Toxicology
2. Biochemical Pharmacology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Metabolism of Sialic Acids

Principal Investigator: Leonard Warren

Other Investigators: Mrs. Smith (part-time)

Man Years (calendar year 1958):
Total: 1-1/3
Professional: 1
Other: 1/3

Project Description:

Objectives: To study the intermediary metabolism and chemical properties of sialic acids in order to understand its role in physiological and pathological states.

Major Findings:

1. A new assay for sialic acid based upon the coupling of the periodate oxidation products of sialic acid with thiobarbituric acid. This thiobarbituric acid assay is, at the present time, 5 times as sensitive as any of the known tests. There is reason to believe that this test may become almost 20 times as sensitive. This assay should be more specific for sialic acid than any of the other assays.

2. Sialic acid in Human Semen and the Male Genital Tract. A small but significant difference in the sialic acid content of normal and subnormal human semen specimens has been found. The normal sialic acid concentrations of human semen, prostatic and seminal vesicle fluids have been established. The sialic acid concentration of human seminal vesicle fluid is higher than in any known mammalian tissue or secretion. From this data it has been calculated that human semen consists of two parts seminal vesicle fluid and one part prostatic secretion. Small amounts of sialic acid are found in human spermatozoa. Sialic acid assays, with special reference to biological secretion such as semen, have been studied and compared.

Part B included [X] Yes [ ] No

3. Sialic Acid in Ova. We have found relatively large amounts of sialic acid in trout ova. At least 50 per cent of this sialic acid is apparently free--unlike all other known forms in which it is bound to mucopolysaccharide or lactose. Sialic acid leaks out of ova suspended in distilled water only when either magnesium, calcium, sodium, or potassium ions are present singly. If any two of these ions are present sialic acid loss is inhibited. The addition of fresh or boiled trout semen to the ova also causes a release of sialic acid.

4. Chemical synthesis of neuraminic acid may have been accomplished. The product has been purified on a cellulose column and is now being characterised. Unsubstituted neuraminic acid should be of considerable chemical and metabolic significance.

Significance to NIAMD Research: Sialic acid is found in relatively large amounts in many mucopolysaccharides. Little is known of its function. The study of sialic acid and its metabolism is of special relevance to an understanding of rheumatic processes, cystic fibrosis of the pancreas, fertilisation, nerve function and many other fields.

Proposed Course of Projects: To complete and extend investigations listed.

IMS-MIK
Individual Project Report
Calendar Year 1958

Part B: Publications

Publications other than abstracts from this project:

(1) Warren, Leonard. Sialic acid in human semen and in the male genital tract. J. Clin. Invest., in press.

Serial No. NIAMD-_ 99
1. Pharmacology & Toxicology
2. Biochemical Pharmacology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Histidine, Histamine & Related Imidazoles & Amines

Principal Investigator: Herbert Tabor

Other Investigators: B. Ames, H. Bauer, G. Crowley, J. Wolff, and L. Wyngarden

Man Years (calendar year 1958):
Total: 4-1/2
Professional: 3
Other: 1-1/2

Project Description:

Objectives: To study the biosynthesis, intermediary metabolism, and pharmacological activity of these compounds in order to understand better their physiological and pathological role.

Major Findings: Work has been continued on the characterization of the following enzymes from liver concerned with the metabolism of formiminoglutamic acid.

(1) Formimino-glutamic acid + tetrahydrofolic acid ⇌ Formiminotetrahydrofolic acid + glutamic acid

(2) Formiminotetrahydrofolic acid → 5,10-methenyltetrahydrofolic acid + NH_3

(3) 5,10-Methenyl-tetrahydrofolic acid ⇌ 10-formyltetrahydrofolic acid

The characteristics of each enzyme has been studied. Of particular note is the marked stimulation of enzyme II by K^+ or NH_4^+, and the reversibility of enzyme III. The latter is particularly significant since the reversibility of enzyme III is apparently necessary for the incorporation of formate into serine, etc.

Imidazoleacetic acid riboside has been synthesized, and shown to be identical with the material excreted in the urine after imidazoleacetic acid administration.

Part B included [X] Yes [ ] No

Further studies on histidine biosynthesis have also been carried out. The various histidine requiring mutants of Salmonella typhimurium have been examined for the enzymes of histidine biosynthesis. Support has been obtained for the ideas that the mutants are missing various enzymes in the pathway and that the genes are linked in the order of the biosynthesis (in collaboration with Dr. P. Hartman). It has been found that the bacteria do not synthesize the various biosynthetic enzymes when histidine is present in the medium. Under certain conditions of growth on limiting histidine it has been possible to raise the levels of enzymes 20 or 30 fold over that of the wild-type bacteria.

Significance to NIAMD Research: Histidine is an essential amino acid, and its products and derivatives enter into many important metabolic relationships. The C-2 of the imidazole ring enters into the "one carbon" pool, and thus these studies are closely related to other studies on the role of folic acid and vitamin B-12 carried out in this laboratory and elsewhere in NIAMD.

Imidazoleacetic acid riboside is of significance in that it represents a new kind of natural riboside. It has also been used elsewhere as a useful tool as an in vivo trapping agent for ribose.

Proposed Course of Project:

I. Histidine Metabolism and Related Studies on Folic Acid-Catalyzed Reactions

1. Further characterization of cyclohydrolase (enzyme III) from liver. Purification of Pseudomonas enzymes that appear to have a different pathway for formiminoglutamic acid degradation. Further studies on precursors of formiminoglutamic acid, namely imidazolonepropionic acid.

2. Studies on the possible role of formimino-transfer reactions in other reactions. Studies on the role of B-12 in various reactions.

3. Studies on mechanism of aminopterin inhibition, with particular emphasis on the question of drug adaptation.

4. Biological syntheses and degradation of carnosine, anserine, ergothioneine, and other related imidazoles. Enzymatic synthesis of imidazoleacetic acid riboside.

NIH-NIH
Individual Project Report
Calendar Year 1958

Part B: Publications

Publications other than abstracts from this project:

(1) Tabor, H., and Wyngarden, L.: A method for the determination of formiminoglutamic acid in urine. J. Clin. Invest. 37: 824-828, 1958.

(2) Hiatt, H. H., Goldstein, M., and Tabor, H.: Urinary excretion of formiminoglutamic acid by human subjects after antifolic acid therapy. J. Clin. Invest. 37: 829-832, 1958.

(3) Rabinowitz, J. C., and Tabor, H.: The urinary excretion of formic acid and formiminoglutamic acid in folic acid deficiency. J. Biol. Chem. 233: 252-255, 1958.

(4) Bauer, H.: Confirmation of the structure of imidazoleacetic acid riboside by synthesis. Biochim. Biophys. Acta 30: 219, 1958.

Serial No. NIAMD- 94
1. Pharmacology & Toxicology
2. Biochemical Pharmacology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Enzyme and Endocrine Studies on Tryptophan and Nicotinic Acid Metabolism

Principal Investigator: Alan H. Mehler

Other Investigators: Catherine Rhodes

Man Years (calendar year 1958):
Total: 1-1/3
Professional: 1/2
Other: 5/6

Project Description:

Objectives: To isolate the individual steps in the sequence of reactions resulting in nicotinic acid formation, to study the properties of the enzymes involved, and to describe the intermediate metabolites. With the reactions available, to study the relation of these enzymes to altered metabolic conditions.

Methods Employed: Enzymes are obtained from various sources and purified by the variety of methods currently used in this field. Chemical and physical, especially spectrophotometric, methods are used to measure enzyme activity and to identify products. Possible substrates and products are synthesized by conventional organic chemical techniques. Isotopic compounds are synthesized and radioactivity is measured to follow the course of reactions *in vivo* and *in vitro*. Animals are treated to produce altered metabolic states, and enzymes from such animals are assayed. Methods have been adapted to assay enzymes in tissue culture and organ culture preparations.

Major Findings: The nature of the interaction of endocrine factors on the control of the level of picolinic carboxylase in liver has been explored further in collaboration with Mr. McDaniel. The enzyme level is increased through an effect of cortisone, and this effect is opposed by the combined effects of insulin and growth hormone, but not by either alone. The cortisone effect is also opposed by thyroxin.

Part B included [X] Yes [ ] No

3-Hydroxyanthranilic oxidase, found in normal livers at high levels, could not be detected in several strains of tissue culture cells, presumed to be mouse liver cells. The enzyme was also not found in a solid tumor derived from one of the tissue culture lines, but was found in a mouse hepatoma that had been transplanted subcutaneously for several years. The tissue culture cells and tumors were provided by Dr. Virginia Evans of the National Cancer Institute.

Studies on the chemical reactions of the unstable product of 3-hydroxyanthranilic acid oxidation have added support for the proposed structure: 2-amino-3-carboxy-5-formyl-2,4-trans,cis-pentanoic acid.

Isotope trapping experiments with ring-labeled 3-hydroxyanthranilic acid showed conversion to quinolinic acid but not to picolinic acid in normal rats.

Significance to NIAMD Research: Two lines of inquiry are related to NIAMD research. One is a study of the reactions that influence niacin metabolism in order to gain more insight into the biochemistry of this vitamin. The other is the analysis of the effect of hormones on liver enzymes, which may give information about the nature of the metabolic lesions in diabetes.

Proposed Course of Project: Attempts will be continued to find *in vitro* systems for demonstrating the effects of hormones on the level of picolinic carboxylase. The metabolism of 3-hydroxy-anthranilic acid will be investigated further.

PHS-NIH
Individual Project Report
Calendar Year 1958

Part B: Publications

Publications other than abstracts from this project:

(1) Mehler, Alan H., McDaniel, E. G., and Hundley, James M.: Changes in the enzymatic composition of liver. I. Increase of picolinic carboxylase in diabetes. J. Biol. Chem. 232: 323-330, 1958.

(2) Mehler, Alan H., McDaniel, E. G., and Hundley, James M.: Changes in the enzymatic composition of liver. II. Influence of hormones on picolinic carboxylase and tryptophan peroxidase. J. Biol. Chem. 232: 331-335, 1958.

Serial No. NIAMD- 68
1. Pharmacology & Toxicology
2. Biochemistry of Amino Acids
3. Bethesda

PHS-WTH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: The Localization of Absorbed Antigens by Means of Specific Fluorescent Antibody Globulin

Principal Investigator: Dr. E. W. Emmart

Project I:

The localisation of streptococcal hyaluronidase within the tissues of the mouse, (A) following injection of the enzyme and (B) following injection of washed suspension of streptococci.

Principal Investigator: Dr. E. W. Emmart

Other Investigators: Dr. I. Kialso, Chief Surg. Pathology Branch, Clinical Neuropathology
Mr. W. A. Turner and Mrs. Thelma Mathews
(laboratory aid - 1 hour per day)

Project II:

Comparative study of anti-lens protein sera with normal sera by means of paper strip electrophoresis technique. This protein is especially of interest since it is among the few which are reported to be autoantigenic.

Principal Investigator: Dr. E. W. Emmart

Other Investigators: Dr. J. Miguel, Fellow Neurological Institute
Mr. W. A. Turner

Project III:

Antibodies to alpha lens protein and the tagging of lens protein with specific antisera.

Principal Investigator: Dr. E. W. Emmart

Other Investigators: Dr. R. A. Reenick, Lab. of Ophthalmology, Sect. Chem.

Part B included [X] Yes [ ] No

Man Years (calendar year 1958):
Total: 100%
Professional: 92.4%
Other: 7.6% (Serves on committee for development of standards of rating applicants for positions in biology at N.I.H. and other government agencies.)

Project Description:

Objectives:

1. Studies on hyaluronidase:

These studies on streptococcal hyaluronidase have been undertaken to determine the sites of absorption of this antigenic enzyme. This enzyme, by definition, is one which depolymerises and hydrolyses hyaluronic acid of the ground substance. While this enzyme is not toxic, these changes in the chemistry of the ground substance are precursors to other cellular changes in the tissue matrix. Antibody to the enzyme inhibits this action and it is possible that antibody-antigen complex may be retained within the body and this may itself become an antigen. Since Streptococcal infections in humans are followed by the development of sera high in antibody the study of the absorbed antigen has been undertaken, with the hope that data obtained from the experimental animal may illuminate the course of tissue changes in humans, especially where recurrent synchromes occur.

Where the streptococcal organism itself has been introduced many toxic antigens are involved in the resulting tissue necrosis. Hyaluronidase is observed to be present in tissues along with other enzymes elaborated concurrently.

Methods employed for tissue studies are essentially those of the Coons fluorescent antibody technique using fluorescein isocyanate as a fluorochrome. In addition the invading streptococcal organism has been tagged with lissamine rhodamine B 200 conjugated to group C streptococcal antiserum.

The degeneration of the muscle during infection is being followed by the use of antimyosin sera conjugated to fluorescein.

The studies on electrophoresis of globulin shows the increase in globulins with antibody development.

These projects will be continued in greater detail using high magnification when equipment ordered finally arrives from Germany.

The major findings (1958) are represented in the following papers published in 1958:

NIH-411
Individual Project Report
Calendar Year 1958

Part B: Publications

Publications other than abstracts from this project:

(1) Ewart, E. W.: Observation on the absorption spectra of fluorescein, fluorescein derivatives and conjugates. Archives of Biochemistry and Biophysics 73: 1-8, 1958.

(2) Ewart, E. W., Cole, R. M., May, E. L., and Longley, J. B.: Studies on streptococcal hyaluronidase and antihyaluronidase. II. The localization of sites of absorption of streptococcal hyaluronidase (group C) with fluorescent antibody. J. Histochem. & Cytochem. 6: 161-173, 1958.

(3) Klatso, I., Horvath, B., and Ewart, E. W.: Demonstration of myosin in human striated muscle by fluorescent antibody. Soc. Exp. Biol. & Med. 97: 135-140, 1958.

Serial No. NIAMD- 96
1. Pharmacology & Toxicology
2. Biochemistry of Amino Acids
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Biosynthesis of Gramicidin J

Principal Investigator: Dr. K. Kurahashi

Other Investigators: Mr. Albert Greenfield and Mrs. Akie Sigimura

Man Years (calendar year 1958):
Total: 2-2/3
Professional: 2
Other: 2/3

Project Description:

Objectives:

To elucidate the mechanism of biosynthesis intermediate metabolism, a cyclic hexapeptide, Gramicidin J, which is produced by Bacillus brevis.

Methods Employed:

A cell-free extract of Bacillus brevis was prepared by sonication of cells harvested at the stationary phase of growth. These extracts were incubated with appropriate carbon-14 labeled amino acids in addition to various co-factors. The incorporation of carbon-14 labeled amino acids into Gramicidin J, and possible intermediate substances is detected by the use of paper chromatography, ion exchange resin chromatography and paper electrophoresis.

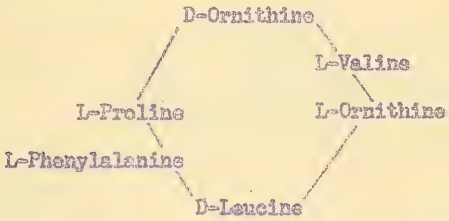
Major Findings:

It was found that a cell-free enzyme solution from B. brevis incorporated C^{14}-phenylalanine into a compound which migrates as a single substance on paper chromatograms with several solvent systems, and on paper electrophoresis at pH 2 and 11. The hydrolysis of this compound gave rise to five amino acids which have Rf values similar to those of leucine, phenylalanine, valine, proline and ornithine on paper chromatograms developed with butanol-acetic acid-water. However, the peptide is not gramicidin J, as indicated by its behavior on paper chromatograms and by paper electrophoresis, as well as by

Part B included [ ] Yes [X] No

the fact that it is not retained by either anion or cation exchange resins when 60 per cent ethanol is used as the solvent. Its amino acid composition suggests that it may be related to the antibiotic substance.

Significance to NIAMD Research: Gramicidin J, an antibiotic produced by Bacillus brevis is a cyclic polypeptide. Its structure has been elucidated by S. Otani and Y. Saito and is illustrated by the following formula:



Since there has been no other detailed work done on polypeptide biosynthesis except that of glutathione by J. E. Snoke et al., a study of the biosynthesis of gramicidin J has been undertaken. The objective of this study is to elucidate whether the biosynthesis of gramicidin J follows the pattern of step-wise formation of polypeptide as is the case in the biosynthesis of glutathione or if it follows the "template mechanism" through the activated amino acids, as has been proposed for protein synthesis. A third alternative is that these polypeptides may be degradative products of large protein molecules.

Proposed Course of Project: The conditions for the formation of the peptide are being studied concurrently with its more rigorous identification.

Serial No. NIAMD- 97
1. Pharmacology & Toxicology
2. Biochemistry of Amino Acids
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: The Biochemistry of Secondary Sulfur-containing Compounds

Principal Investigator: Simon Black

Other Investigators: Miss Blondel Hudson (previously Mrs. Eileen Harte) and Dr. Norman Bauman (previously Dr. Edith Wolff)

Man Years (calendar year 1958)
Total: 3-2/3
Professional: 3
Other: 2/3

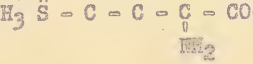
Project Description:

Objectives: A long term objective is discovery of enzymatic mechanisms involved in the synthesis of constituents of living tissue, particularly of proteins.

Methods: Organisms and extracts of organisms are tested for their ability to convert certain sulfur-containing compounds to new substances, or to form or transform known sulfur compounds. Chemical, chromatographic, radiochemical, and radioautographic methods are used. Mechanisms of formation are elucidated by classical enzymological methods.

Major Findings: (1) Yeast has been found to contain two enzyme systems for the reduction of methionine sulfoxides. One system is specific for one of the four isomers, the Ll isomer:

Another reduces the Ld and Dd isomers:



Part B included [X] Yes [ ] No

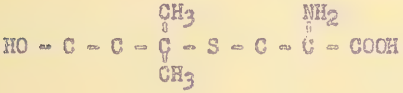
Both systems require TPNH.

The L1-reducing system has been further separated into two fractions, both of which are required for the formation of methionine and for the disappearance of TPNH. The possibility of an intermediate reduction product

is being investigated.

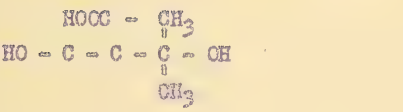
(2) The formation of felinine by cats has been studied by means of radioactive tracer substances.

S^{35} cysteine and C^{14} acetate are incorporated into felinine in substantial amounts. C^{14} valine or serine are not substantially incorporated, however. It is considered possible that felinine is formed from cysteine and some derivative of mevalonic acid which is known to be derived from acetate.

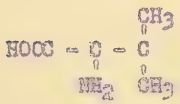


Felinine

Cysteine



Mevalonic acid



Valine

Significance to NIAMD Research: Knowledge of the intimate chemical transformations in living cells will serve as a basis for better understanding of the nature of disease and its more intelligent treatment.

Proposed Course of Project: The immediate plan is to learn the relation of newly found substances and reactions to established biochemical processes.

PHS-NIH
Individual Project Report
Calendar Year 1958

Part B: Publications

Publications other than abstracts from this project:

(1) Wolff, E. C., and Black, S.: Formation of the methylthiol ester of 3-phosphoglyceric acid catalysed by glyceraldehyde-3-phosphate dehydrogenase. Arch. Biochem. & Biophys. (in press).

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

ANNUAL PROJECT REPORT

CALENDAR YEAR 1958

Summary Sheet

LABORATORY OF PHYSICAL BIOLOGY

Estimated Obligations for FY 1959

Total - $1,452,139
Direct - $882,000
Reimbursement - $570,139

Project Numbers 98 thru 116 Included

Serial No. 92
1. Physical Biology
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Effects of hypoxia on physiological and pathological mechanisms in animals.

Principal Investigator: Paul D. Altland

Other Investigators: Edwin C. Thompson, Milton Porter, John Devlin

Cooperating Units: Pathologic studies involving animals exposed to simulated high altitude conducted with cooperation of Dr. Benjamin Highman, Section on Histochemistry (see Serial No. NIAMD-74).

Man Years (calendar year 1958):
Total: 4
Professional: 1
Other: 3

Project Description:

Objectives: (a) To study the effects of repeated exposures to 25,000 and 30,000 feet simulated altitude on dogs with emphasis on analysis of semen quality and structural changes in male reproductive system and on other organ changes at 30,000 feet. Determination of the effect of restraint on altitude tolerance in rats. (b) To study the rate and degree of development of experimental arteriosclerosis in rabbits exposed continuously to an altitude of 16,000 feet. (c) Study of factors effecting blood formation. To study effect of a folic acid antagonist, aminopterin, on blood formation in cold-blooded animals. To study the effects of cobalt feeding of pregnant rats on erythropoiesis in offspring as a means of detecting the presence of erythropoietin.

Methods employed: Altitude exposures conducted in decompression chambers. Physiologic, hematologic and cytologic techniques used.

Major findings: Dogs exposed to 30,000 feet show reduced seminal volume and subnormal spermatozoa counts after 7 exposures. Moderate to severe testis pathology occurs in some dogs after 14 to 29 exposures. No reproductive abnormalities were observed during 54 daily exposures to 25,000 feet. This is in marked contrast to severe testis pathology that develops in rats similarly treated.

Major findings (cont'd):

It was found that restrained young adult male and female rats died significantly sooner than unrestrained controls when exposed acutely to a simulated altitude of 33,500 feet. The colonic temperature of the restrained rats was lower than unrestrained rats. This hypothermia is a possible causal agent in the earlier deaths of restrained rats.

It was found that blood formation in cold-blooded animals is not affected by cobalt and hypoxia and by liver extracts with and without mineral supplement. Anemia and leucopenia in turtles developed following 6 weeks treatment with the folic acid antagonist aminopterin. It is indicated that folic acid is a vitamin involved in blood formation in reptiles.

Significance to NIAMD research: Contributes to an understanding of the differential effect of hypoxia on the reproductive system of animals. Results indicate the importance of evaluating the effects of restraint whenever this method is used as a convenience in handling of animals in experiments. Provides basic data on some of the factors which affect blood formation in cold-blooded animals.

Proposed course of project: Further studies on the mechanisms which influence altitude tolerance. Study of the influence of different degrees of exercise at ground level on the altitude tolerance of rats will be conducted. Studies on the relationship of hypothermia and altitude tolerance will be continued. Experiments will be conducted on the rate and degree of development of experimental arteriosclerosis in chickens exposed continuously to 16,000 feet simulated altitude.

Part B included Yes

PHS-NIH
Individual Project Report
Calendar Year 1958

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Highman, B., Roche, J., and Altland, P. D.: Endocarditis and glomerulonephritis in dogs with aortic insufficiency. AMA Arch. Path. 65: 388-394, 1958.

Altland, P. D. and Thompson, B. C.: Some factors affecting blood fermentation in turtles. Proc. Soc. Exp. Biol. & Med., Nov., 1958 (in press).

Highman, B., Ewing, E. M., and Thompson, B. C.: Serum transaminase and alkaline phosphatase levels after large doses of Norepinephrine and Epinephrine in dogs. Am. J. Physiol. (in press).

Bartlett, Jr., R. G. and Altland, P. D.: Effect of restraint on alkali tolerance in the rat. Accepted for publication in J. Appl. Physiol.

Serial No. 99
1. Physical Biology
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Pulmonary ventilation

Principal Investigator: Roscoe G. Bartlett, Jr.

Other Investigators: Heinz Specht, Howard Brubach, Herman Luebbing, Ernest Kelcy

Cooperating Units: None

Man Years (calendar year 1958):
Total: 7
Professional: 5
Other: 2

Project Description: Studies of pulmonary ventilation and studies on factors affecting body heat balance.

Objectives: The principal objective of the overall project is to explore new phenomena regarding breathing behavior with regard to their physiological significance. In order to accomplish this, various studies of physiology must be undertaken in order to accurately control the experimental situation. The interrelation of work of, and resistance to, breathing have been sought as limiting factors in normal as well as abnormal conditions of breathing. A secondary objective has been the study of adaptation to environmental stresses and factors which modify such adaptive responses. The mechanism of control of heat balance in warm-blooded animals, including man, has a strong bearing on energy budget of individuals under various circumstances.

Methods employed: Subjects are studied for oxygen consumption, carbon dioxide production, breath velocity patterns, and volume measurements as necessary. New devices for several of these measurements were made, including a whole body plethysmograph for the determining of lung airway resistance and a study of eventilatory mass flow of air into the lungs.

Major findings: A broadly applicable method for the quantitative and continuous measurement of lung airway resistance in man was devised by adapting the whole body plethysmograph technique to obviate spurious effects formerly caused by temperature and

Major findings (cont'd):

humidity changes of the exhaled and inhaled breath. The use of air and other respirable gas mixtures at body temperature, saturated with water vapor permits the integration of flow and pressure measurements into resistance values and also allows the demonstration of such minor volume changes as that caused by the heart beat.

A dramatic demonstration was further made possible of the effects caused by the continual influx of oxygen through the lung surface - a phenomenon ordinarily masked by the reciprocal flow of respiratory gases during breathing. This phenomenon was studied under various ventilatory conditions in addition to breath holding where it is directly indicative of the rate of overall oxygen consumption and also during hypo- and hyperventilation where it demonstrated the ratio of carbon dioxide output to oxygen use in a dynamic fashion.

A simple modification of clinical spirometry was demonstrated to permit the measurement of the oxygen cost of the work done in breathing. It was shown that as a machine the lung has an increasing mechanical efficiency as airway resistance increases to a maximum after which the efficiency falls off, but by discounting work done on the lung-chest machine, the work done on the breath was shown to increase with increasing resistance to a maximum and then fall off. At low resistance levels work output was contraction speed limited and at high resistance levels it was contraction-force limited. Maximum effort was shown to incur a consistently constant oxygen cost indicating that all available muscular mechanisms could be brought to bear on ventilation at any resistance level.

Studies on the effect of restraint on body temperature maintenance in various laboratory animals showed that marked effects were induced primarily by the hypothermia which resulted from exposure to a cold environment. In the case of altitude tolerance the body temperature fall was of survival value due to the decreased metabolic activity incident to lowered body temperature. A general warning regarding the unwitting use of restraint for convenience of studying animal physiology is supported by the various demonstrations of this effect.

Significance to NIAMD research: The primary studies are related to the better understanding of pulmonary function and are expected to result in the more adequate quantitative measurement of the efficiency of this function in health and disease.

Proposed course of project: It is planned to renew studies on the effects of density of respired gases on breathing patterns and to attack the problems of measurement of functional alveolar area.

Part B included Yes

PHS-416
Individual Project Report
Calendar Year 1958

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Bartlett, R. G., Jr., Brubach, H. F., and Specht, H.: Demonstration of ventilatory mass flow (AVMF) during ventilation and apnea in man. Accepted for publication by J. Applied Physiol.

Bartlett, R. G., Jr. and Young, H. W.: Free running in the Albino rat and its effect on restraint hypothermia. Accepted for publication by J. Applied Physiol.

Bartlett, R. G., Jr., Brubach, H. F., Trimble, R. G., and Specht, H.: A broadly applicable method for airway resistance measurement in man. Accepted for publication by J. Applied Physiol.

Bartlett, R. G., Jr.: Effects of restraint on oxygen consumption of the cold exposed guinea pig. Accepted for publication by J. Applied Physiol.

Bartlett, R. G., Jr., Brubach, H. F., Trimble, R. G., and Specht, H.: Relation of increased airway resistance to breathing work and breath velocity and acceleration patterns with maximum and near maximum breathing effort. J. Applied Physiol., 13: (1) 194-204, Sept., 1958.

Bartlett, R. G., Jr., Mantel, N., Foster, G. L., and Bernstein, L.: Core to surface thermal gradients in the rat at several environmental temperatures. Am. J. Physiol., 193: (3) 541-546, June, 1958.

Serial No. 100
1. Physical Biology
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Insect or Invertebrate Metabolism

Principal Investigator: John B. Buck

Other Investigators: Lee Levenbook, Margaret L. Keister, Helen D. Park, Christyna E. Macca

Cooperating Units: None

Man Years (calendar year 1958):
Total: 5
Professional: 4
Other: 1

Project Description:

Objectives: See "Proposed Course of Project".

Methods employed: Carbohydrate metabolism has been followed by chromatographic methods of identification and assay of various types of blood sugars, chemical isolation of tissues of glycogen, and respirometry of intact organisms and of tissues. Enzymatic pathways have been followed by use of radioactive tracers. The aerobic-anaerobic transition zone is being studied by measuring oxygen uptake before, during and after exposure to various partial pressures of oxygen. The biophysical aspects of gas transfer involve dimensional study of the respiratory system, respirometry and computation. Electrometric methods were applied to an intensive study of neurogenic initiation of bioluminescence in fireflies.

Major findings: Dr. Park is preparing for publication her study of fly pupal respiration. In addition to findings reported last year she has shown that an oxygen debt is incurred during 4 hours of anoxia and that there is greater repayment in pure O_2 than in air (i.e. there is physical limitation to O_2 uptake). Dr. Keister has completed a roughly parallel study of fly larvae and has begun work on adult flies. In these she has already found a very marked rise in metabolic rate occurring rather suddenly at an age of two to three days and that respiration may rise to 20 or more times the resting level (i.e. to the flight level) without overt activity. A long-lasting depression of respiration by brief exposure to CO_2 has also been discovered.

Major findings (cont'd):

Dr. Buck is preparing for publication a report on joint work with Dr. James Case of the University of Iowa on the control of flashing in the firefly - a problem of great interest because of the many similarities between nerve-muscle and the neuro-photogenic system. Among physiological parameters measured are (1) response latency as a function of temperature, O_2 tension, stimulus strength, stimulus duration, stimulus frequency, organ structure (2) flash intensity as a function of the above variables, (3) nerve conduction rate under various conditions. Recruitment and coordination of photogenic cells has been studied in waxing experiments and direct and neural stimulation paths have been compared. In addition, direct recording of the cord action potentials has demonstrated for the first time that the spontaneous flash is directly excited by neural activity.

Significance to NIAMD research: The studies on intermediary metabolism are specifically oriented to contribute to the Institute's diabetes program. It is hoped that they may shed light on the basic mechanism of glycogen mobilization. The anoxia study has already indicated that insects may show interesting differences from mammals in regard to oxygen debt accumulation and anaerobic metabolism. The biophysical study, it is hoped, can contribute to the theoretical knowledge of gas transfer mechanisms in respiratory systems.

Proposed course of project: Our unit is continuing work on the physiology and biophysics of insect respiration as a primary objective, but staff interest suggests that there will be some diversification of pathways in the future. Thus, (a) Dr. Wolf, having brought to a close her study of fly pupal respiration in relation to O_2 tension, wishes to resume work on flight; specifically to devise tissue culture methods for use in studying growth and differentiation. (b) Dr. Lee Levenbook has replaced Dr. Stanley Friedman in our unit, pursuing a study of biochemical activity from carbohydrate metabolism to protein synthesis. (c) Dr. Buck has now spent two summers in collaborative work in insect electrophysiology (see report below) and it is likely that electrophysiological techniques may enter into our future respiration studies both in the role of stimulation of (flight) metabolism and in recording respiratory rate responses.

Part B included Yes

PHS-413
Individual Project Report
Calendar Year 1958

Part B: Honors, Awards, and Publications

Buck, John B.: Cyclic CO_2 release in insects. IV. A theory of mechanism. Biol. Bull., 114: (2) 118-140, April, 1958.

Buck, John B.: Book review "Light on Light" of a HISTORY OF LUMINESCENCE. Quart. Rev. Biol., 33: 59-61, May, 1958.

Buck, John: Possible mechanism and rationale of cyclic CO_2 retention by insects. Proc. 10th International Congress of Entomology, 2: 339-342, Nov., 1958.

Buck, John and Friedman, S.: Cyclic CO_2 release in diapausing pupae. III. CO_2 capacity of the blood: carbonic anhydrase. J. Insect Physiol., 2: (1) 52-60, March, 1958.

Buck, John and Keister, M.: Cyclic CO_2 release in diapausing pupae. II. Further data. J. Insect Physiol., 1: 327-340, 1958.

Fraenkel, G. S. and Friedman, S. Carnitine. Vitamins & Hormones. 15: 73-118, Academic Press, New York, 1957.

Friedman, Stanley: Sustained flight in Rhodnius (by a new method) and its effect on blood pH. Accepted by J. Insect Physiol.

Friedman, S. Determination of carnitine in biological materials. Arch. Biochem. & Biophys., 75: (1) 24-30, May, 1958.

McDermott, F. A. and Buck, J. B.: The lampyrid fireflies of Jamaica. Accepted by Transactions of the Am. Entomological Soc.

Park, Helen D.: Sensitivity of hydra tissues to X-rays. Physiological Zoology, 31: (3) 188-193, July, 1958.

Serial No. 101
1. Physical Biology
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Mechanism of the circulatory reaction of sensitive species to synthetic macromolecules.

Principal Investigator: Louise H. Marshall

Other Investigators: Charles H. Hanna

Cooperating Units: None

Man Years (calendar year 1958):
Total: 3
Professional: 1
Other: 2

Project description:

Objectives: To determine the cause of vasodepression and edema in rats after dextran administration and in dogs after polyvinylpyrrolidone (PVP) by studying their appearance in conscious animals, the refractory condition, and the age at which they occur.

Methods employed: Arterial blood pressure recordings from conscious rats bearing indwelling aortic cannulae, and dye (T-1824) extravasation, demonstrated the systemic hemodynamic and local permeability effects respectively. The percentage of disrupted mast cells, the most important source of histamine in rats, stained by Pugh's technique was determined in peritoneal washes and mesentery spreads. The uteri of pregnant rats were exposed under anesthesia and dextran injected into the fetuses; survival to term and after was surprisingly good. The isolated muscle strip technique was used to test whether refractoriness could be produced in vitro. Uterine strips of rats sensitized with stilbestrol were found to be sensitive to dextran. Newborn puppies and rats were injected with PVP and dextran respectively at various ages because of the possibility of correlating reactiveness with the appearance of histamine and serotonin.

Major findings: In conscious rats the systemic reaction to dextran consisted of slowly developing vasodepression without the temporary recovery seen during barbiturate anesthesia. Pretreatment with various histamine or serotonin releasers or antagonists abolished the vasodepression after dextran and lessened the incidence of edema. The percentage of disrupted mast cells in peritoneal washes and mesentery spreads did not correlate with each other or with the reaction of rats to dextran or other histamine releasers. Fetal administration of dextran did not alter rats subsequent sensitivity. The contraction height of sensitized rat uterine strips did not diminish with successive applications of dextran. Rats became sensitive to dextran at about one week of age, puppies to PVP between the 29th and 31st day.

Significance to NIAMD research: The investigations described constitute research in the basic physiology of cellular sensitivity.

Proposed course of the project: To follow the promising leads until the mechanism of the reaction is more fully understood.

Part B included Yes

PHS-NIH
Individual Project Report
Calendar Year 1958

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Marshall, L. H. and C. M. Hamma: Circulatory reaction of rats and dogs to dextran or polyvinylpyrrolidone after 48/80. Proc. Soc. Exptl. Biol. Med. 97: 587-590, 1958.

Serial No. NIAMD 102
1. Physical Biology
2. Physical Biochemistry
3. Bethesda

Part A.

Project Title: Immunochemical approaches to the isolation and characterization of proteins.

Principal Investigator: R. R. Williams

Other Investigators: L. C. Stewart, J. C. Jenkins, and C. Isreal

Cooperating Units:
Dr. Kurt Bloch and Dr. J. J. Bunim, CI-NIAMD (Serial No. 118-C)
Dr. E. M. Lerner, LPH-NIAMD (Serial No. 76)

Man Years (Calendar year 1958): Total: 4
Professional: 2
Other: 2

Project Description:

Objectives:

The immunochemical approach, because of the structural specificity of antibodies, presents some unique opportunities in the approach to the isolation and intimate structural characterization of proteins.

Under investigation are the group of proteins involved in the sensitized sheep cell agglutination activity of rheumatoid arthritic serum. Also, an immuno-chromatographic approach to the structure of peptides involved in the antigenic sites of bovine serum albumin is being attempted.

Methods:

Beside the usual immunochemical methods, a new method using a virus coated with various proteins shows promise of becoming a valuable tool. The other usual methods of protein characterization are also used.

Patient Material: See reports of Drs. Bunim and Bloch.

Major Findings:

(1) A protein preparation, almost homogeneous in the ultracentrifuge, has been isolated from rheumatoid arthritic serum. Precise data on the specific activity of the material has been obtained.

(2) In collaboration with Dr. Edwin M. Lerner, the presence of sensitized sheep cell agglutinating activity has been demonstrated in the serum of rats infected with a strain of streptobacillus moniliformis which produces joint lesions.

(3) Correlation of the quantitative sensitized Newcastle hemolysis inhibitor activity with clinical rheumatoid arthritis has been established and extended.

Significance:

The precise specific activity data now available on our purified rheumatoid factor preparations provides a solid basis for further purification steps. Evidence has been accumulated which indicates only part of the macroglobulin molecules in rheumatoid or normal serum participate in the sensitized sheep cell reaction. The demonstration of a similar activity in rat serum from streptobacillus moniliformis infected rats provides an excellent experimental model for the production of this activity. Possibly it will also provide some sort of model for rheumatoid arthritis.

Proposed Course of Project:

(1) Further purification of present rheumatoid factor preparations.

(2) Characterization of the protein involved by amino acid analysis, end group studies, possibly work on active binding sites.

(3) Isolation from animal sera of proteins with similar activity.

(4) Attempt to characterize antigenic binding sites of bovine serum albumin. This may aid in item 2.

(5) In collaboration with Dr. K. J. Bloch, studies of the genetic distribution of rheumatoid factor in relatives of patients with the disease.

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Lerner, E. M. II, Williams, R. R., and Jenkins, J. C.: Sensitised sheep cell hemagglutination reaction in rats with experimental infection of bone and joint. Proc. Soc. Exp. Biol. and Med. 99, 249, 1958.

2. Williams, R. R., Stewart, L. C., and Jenkins, J. C.: Isolation and purification of the rheumatoid factor. Proc. Soc. Exp. Biol. and Med., in press.

Individual Project Report
Calendar Year 1958

Serial No. NIAMD- 109
1. Physical Biology
2. Physical Biochemistry
3. Bethesda

Part A.

Project Title: The mechanism of the orderly aggregation of proteins in building up biological structures.

Principal Investigators: Dr. Kolcman Laki and Dr. H. A. Saroff

Other Investigators:

Dr. W. J. Bowen	M. L. Martin
Dr. W. R. Carroll	M. J. Callanan
Dr. D. R. Kemins	J. F. Cairns
Dr. F. Irreverre	J. W. Healy
Dr. J. A. Gladner	E. R. Mitchell
Dr. R. B. Simpson	E. N. Smith
Dr. F. M. Saad	W. L. Conway
	E. F. Wilson

Cooperating Units:
Dr. J. O. Davis, LKEM-NIH (Serial No. _____)
Dr. E. Weinbach, LTD-NIAID (Serial No. _____)
Dr. J. E. Folk, OBC-NIDR (Serial No. _____)
Mr. P. Pronove, GMET-NHI (Serial No. _____)
Dr. E. Mihalyi, LCPM-NHI (Serial No. _____)
Dr. B. Horvath, MN-NINDB (Serial No. _____)
Dr. Leo Mandelkern, Nat'l. Bur. of Standards
Dr. F. C. Bartter, GMET-NHI (Serial No. _____)
Dr. Sam Asen, Crops Res. Div., Agri. Res. Service, U. S. Dept. of Agriculture
Dr. J. F. Thompson, U. S. Plant, Soil, and Nutrition Laboratory, Agricultural Research Service, U.S. Dept. of Agriculture

Man Years (calendar year 1958): Total: 16-1/3
Professional: 11-1/3
Other: 5

Project Description:

Objectives:

The objective of this project is the same as has been outlined in 1957: A study of the polymerisation of proteins in order to gain insight as to how cells build up network structure such as the

Objectives: (Cont'd.)

structures involved in muscular contraction and blood coagulation. The main part of this project is, therefore, devoted to the study of proteins involved in muscular contraction and blood coagulation. The main part of this project is, therefore, devoted to the study of proteins involved in muscular contraction and blood coagulation.

Methods:

The method of attack is both direct and indirect. In the direct attack the proteins of muscular contraction and blood coagulation are separated from their native milieu and are studied under arbitrary conditions which are selected to reveal properties of interest. In the indirect attack studies are made on some other (already better) known proteins to gain information before the direct attack is made.

In these studies the procedures of biochemistry and physical chemistry are employed. For example: paper and ion-exchange chromatography, enzymology, ultracentrifugal analysis, osmometry, light-scattering measurements, electrophoresis, diffusion measurements, X-ray diffraction, etc.

Major Findings:

It is now becoming increasingly apparent that without the understanding of the detailed composition of myosin, its role in muscular contraction cannot be evaluated.

With the use of metal cyanides, it is now possible to break up myosin into its constituent protein component. So far, 2% rabbit, and crayfish myosin has been tentatively identified as tropomyosin A and 12% of rabbit myosin as the "carbonate subunit." Work is in progress to identify another large component of myosin as actin (Komins, Laki, Carroll, Mitchell).

"The X-ray structure of myosin and its subunit are under investigation. (Laki in cooperation with Mandelkern at the National Bureau of Standards.)

The changes of X-ray patterns during ATP and mercury potassium iodide induced contraction is also under investigation (Laki, Bowen, Mandelkern).

In order to determine molecular weight, an extensive series of measurements of the diffusion and sedimentation constants of

Major Findings: (Cont'd.)

myosin prepared from normal and failing hearts of dogs is being carried out. Preliminary results show no differences in behavior (Carroll, Mitchell in cooperation with Dr. J. O. Davis).

It has been found that the amount of work fiber-bundles of glycerol-treated muscle can perform is not correlated with the energy theoretically available from the splitting of the terminal phosphate of ATP (Bowen, Martin).

The azo dye chrome fast Blue BG has been adapted for the analysis of magnesium in blood sera without the removal of calcium (Bowen, Martin).

Studies for the localisation of the phosphate bound to myosin are further pursued. Preliminary data indicate that part of phosphate is bound to the amino acid serine (Laki, Mihalyi, Knoller).

Taking advantage of the inactivation of thrombin by diisopropylphosphorofluoridate (DFP), radioactive DIP^{32}-thrombin was degraded by mild acid hydrolysis, the radioactive peptides isolated and purified by ion exchange chromatography. It was shown that the radioactive phosphate was bound to serine, located in the sequence: gly.asp.ser.PO_4.gly. This investigation establishes identical sequences at the "active site" in a number of different enzymes and clearly implies that a specific serine residue participates in the hydrolytic activity of these enzymes (Gladner, Laki).

Reasoning from the general thermodynamic properties of ring formation in such compounds as creatinine and glycocyamidine, the acyl shift in proteins has been analysed. The oxazoline and thiazoline ring systems were compared with the creatinine and glycocyamidine systems. A new mechanism for the aggregation of proteins and a new mechanism for general proteolytic enzymatic action has also been proposed based on the acyl shift (Saroff).

This proposed mechanism may turn out to be the first case where we understand the mechanism of enzyme action on the molecular level.

Studies are also being continued on the sulfhydryl group in proteins and the role of the thiazoline ring in determining the sulfhydryl content (Simpson, Saroff).

A more rapid procedure for the isolation and purification of the peptides released from fibrinogen during clotting has been developed.

Major Findings: (Cont'd.)

With the pure peptides at hand it was possible to determine their sedimentation and diffusion coefficients (Carroll, Gladner, Folk, Laki).

Partial structural analysis of these peptides have disclosed a unique make-up: All the negative charges are clustered in the N-terminal vicinity of the molecules. This structure may be of twofold significance: (1) it may specifically direct thrombin to a definite point of hydrolysis on the fibrinogen molecule and (2) the peptides being part of the fibrinogen molecule and thus imparting a region of high local negativity in the molecule may be the means by which fibrinogen molecules repel one another and keep fibrinogen molecules from clotting. Complete structural analysis of peptides A and B is in progress (Gladner, Folk, Laki).

We are now turning our attention to the clotting of human fibrinogen.

Further purification of carboxypeptidase B (100 fold) reported last year and its pro-enzyme has been obtained. Kinetic studies show activation of this enzyme by certain divalent ions (Co^{++} in particular). Carboxypeptidase A also shows a twofold Co^{++} activation (Gladner, Folk).

Some characterization studies of the physical chemical properties of carboxypeptidase B have been carried out, and these are now being extended to the zymogen isolated from pancreas (Carroll, Gladner, Folk).

Studies on the modification of proteins have been continued. The reaction of the amino group of proteins and amino acids with S-methylisothionitrourea has been found to yield the non-polar nitroguanidine group under mild reaction conditions. It has been found that quantitative measurement of this change can be made by following the ultraviolet absorption at 270 mμ (Saroff, Evans).

Model compounds such as hexylamine, decylamine, and dodecylamine have been employed in the past year for the study of anion binding to the amino group. In this class of compounds there is the possibility of evaluating the strength of binding as well as the general electrostatic effects (Saroff, Healy).

In the studies of the binding of anions and cations to proteins, increased emphasis has been placed on searching for specific steric effects in these interactions.

Major Findings: (Cont'd.)

A continuing study of the protamine, salmine, has demonstrated a unique type of ion binding involving 6 or 7 of the 18 possible sites for anions. These 6 or 7 sites bind chloride, thiocyanate, and sulfate very strongly. Since one anion is bound per site, the resulting salmine ion has a charge of +12 in the case of chloride or thiocyanate but only +6 in the case of the sulfate. The influence of this charge on some of the hydrodynamic properties of the molecule has been studied, and the effect of charge and extra salt on sedimentation behavior has been found to be predicted very well by a recent theory proposed by K. O. Pedersen. The effect of the different charges is also apparent in the viscosity behavior (Carroll, Saroff, Mitchell, Callanan).

Studies were continued on the excretion of hydroxyproline with the aim of learning more about the metabolism of the protein collagen. Two pathologic cases were found with a high excretion of bound hydroxyproline into the urine: (1) Marfan syndrome, (2) Touraine-Solente-Jole syndrome. This finding suggests that in these 2 cases there may be a disturbance in the metabolism of collagen. (Irreverre, Pronove, Hayden.)

Efforts were made to isolate hydroxyproline peptides which we found in the urines as reported in previous report. We have now found a color reaction for one of the peptides using β-naphthoquinone-4-sodium-sulfonate. (Irreverre).

Since our previous studies on the hydrolysis of α-guanidino-carboxylic acids by 6N HCl at 110° revealed the formation of cyclic structures to the extent of 50-80%, we turned next to explore the use of nonspecific proteolytic enzymes as a method for hydrolyzing chemically altered proteins and peptides such as the guanidinated ones, thereby revealing the reactive alpha and epsilon amino groups. Preliminary experiments using pancreatin preparation showed encouraging results. (Irreverre).

Chromatographic analysis of the urine of some patients revealed an abnormally high excretion of glycine. Plasma glycine was also found elevated indicating that it is not due to kidney defect but a possible disturbance in glycine metabolism. (Irreverre, in cooperation with Pronove and Bartter).

β-aminoisobutyric acid from the bulbs of Iris Tingitana has been isolated and characterized. (Irreverre, Asen and Thompson).

Significance:

When part of the protoplasm or the whole cell (as in, e.g., cell division or muscular contraction) performs mechanical work, a network structure is built up at least temporarily, mainly through an orderly polymerization of globular proteins. This structure then reacts with the surrounding medium and by utilizing metabolic energy (stored in ATP e.g.) performs work (muscular contraction, amoeboid movement). In order to understand this "mechano-chemical coupling" (the interaction of structure with the surrounding) and its disorders, we must know how such structures are built up. In addition we also have to know the detailed structure of the "building stones," the structure of the polymerising proteins. Muscular contraction and blood coagulation are examples of processes where structures are built up through protein polymerisation. Such knowledge eventually will lead us to the understanding of certain diseases of muscle. Study of blood clotting, in addition to supplying clues for protein polymerization, gives us better understanding of the disorders of blood clotting.

When both direct and indirect approach leads to some specific disease (e.g. hemophilia, rheumatoid arthritis) the advantage offered by studying the disease is utilized to the extent profitable.

Proposed Course of Project:

In the next calendar year the project will be pursued along the lines presented above. The following is intended to be an illustration.

The splitting up of the myosin molecule by various methods will be further investigated.

An attempt will be made to coordinate the X-ray pattern of glycerol-treated muscle fibers with the extent of shortening.

The amino acid sequence of the peptides released during clotting of bovine and human fibrinogen will be further elucidated.

One of these peptides from fibrinogen appears to have smooth muscle contracting effect. This observation will be studied.

Further efforts will be made to purify the pro-enzyme of carboxypeptidase B and study the molecular aspects of its activation.

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

1. Weinbach, E. C., and Bowen, W. J.: The effect of pentachlorophenol on the adenosinetriphosphatase and contractile properties of psoas muscle preparations. Biochim. Biophys. Acta 30 (3), 476-484, Dec., 1958.

2. Bowen, W. J., and Martin, H. L.: The rate of dephosphorylation of adenosinetriphosphate and the isotonic and isometric contraction of glycerol-treated muscle. Am. J. Physiol. 195, 311-315, Nov., 1958.

3. Folk, J. E., and Gladner, Jules A.: Carboxypeptidase B. III. Specific esterase activity. Biochim. Biophys. Acta, in press.

4. Gladner, Jules A., Folk, J. E., Laki, K., and Carroll, W. R.: Thrombin induced formation of co-fibrin. I. Isolation, purification and characterization of co-fibrin. J. Biol. Chem., in press.

5. Gladner, Jules A., Folk, J. E., and Laki, K.: Thrombin induced formation of co-fibrin. II. Preliminary amino acid sequence studies on peptides A and B. J. Biol. Chem., in press.

6. Laki, K., Gladner, Jules A., Folk, J. E., and Kominz, D. R.: The mode of action of thrombin. Thrombosis et diathesis haemorrhagica 2, No. 3/4, 205-217, Sept., 1958.

7. Gladner, Jules A., and Laki, K.: The active site of thrombin, J. Am. Chem. Soc. 80, 1263, 1958.

8. Laki, K., Horvath, B., Klatzo, I.: On the relationship between myosin and tropomyosin A. Biochim. Biophysica Acta 28, 636-637, 1958.

9. Laki, K.: Nature of paramyosins. Science 128 (3329), 683, Sept. 19, 1958.

10. Kominz, D. R., Carroll, W. R., Smith, E. N., and Mitchell, E. R.: A subunit of myosin. Arch. Biochem. Biophys., in press.

11. Saad, F., Kominz, D. R., and Laki, K.: A study of the tropomyosins of three cold-blooded vertebrates of different classes. J. Biol. Chem., in press.

12. Asen, S., Thompson, J. F., Morris, C. J., and Irreverre, F.: Isolation of β-aminoisobutyric acid from bulbs of Iris tingitana var. Wedgewood. J. Biol. Chem., to appear Jan. 1959.

13. Sullivan, M. X., and Irreverre, F.: A highly specific test for creatinine. J. Biol. Chem. 232, 530-534, Aug. 1958.

Serial No. 104
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Investigation of the macromolecular organisation of living matter.

Principal Investigator: Ralph W. G. Wyckoff

Other Investigators: L. W. Labaw, V. M. Mosley; Kozo Momoki and G. Ceolin (Visiting Scientists)

Cooperation Units: Pierre Lépine, Institut Pasteur, Paris, France; D. B. Scott, National Institute of Dental Research (See Serial No. _____).

Man Years (calendar year 1958):
Total: 8
Professional: 5
Other: 3

Project Description:

Objectives: To gain information about the macromolecules that are essential constituents of living matter, to see how they are arranged in the structures they form and to see how this arrangement is altered by infectious and degenerative disease. To study certain of these macromolecules (such as viruses) in purified form after isolation from the living material. The improvement of resolution and the interpretation of highest magnifications of the electron microscope are ancillary objectives.

Methods Employed: The electron microscopy of microorganisms, cells and tissues in suspension or thinly sectioned; the physicochemical characterization of macromolecular components isolated from such material using electron microscopy, X-ray diffraction, and similar established techniques; the development of new physical procedures, including X-ray microscopy, to further such characterization.

Major Findings: The work done in Bethesda during the last year has proceeded along the following main lines:

(1) During the last year there has been a steady extension of the electron microscopy at very high resolution which was begun two years ago for the direct photography of molecular separations in crystals of organic compounds of comparatively low molecular weight (500-700). Using an old electron microscope, modified here for this work, molecular spacings have been recorded down to about 9 angstroms from several chemically related indanthrene derivatives. This work is being actively pushed to provide an adequate experimental basis for an explanation of this close approach to the photography of atomic detail; it also seeks to ascertain the best way to exploit this new procedure for examining organic molecules. A series of X-ray diffraction measurements is being continued on these same compounds to provide data essential to the full interpretation of what the microscope reveals.

(2) Cooperative electron microscopy with the Pasteur Institute designed to determine how a number of typical viruses develop within infected tissues is continuing. During the past year the senior investigator worked in the Pasteur Institute with Dr. Pierre Lépine and his staff on the morphogenesis of the virus of vaccinia as compared to that of fowl pox. An intermediate form as seen in hog pox is under study to further elucidate the structural relationships of the various steps of development.

(3) Special attention continued to be given this year to the development of X-ray microscopic methods directed towards the identification of chemical elements present in a sample through their characteristic X-ray absorption and the fluorescent X-rays they emit. These methods are being applied with increasing success to elements of medium atomic weights and this success is leading to their gradual extension towards those lightest elements which are of maximum biological interest.

(4) The collaborative studies with the Dental Institute designed to apply our new physical methods to the study of teeth and their development is being continued. During the last year this has been mainly in the direction of exploring the further applications to dental problems of our expanding X-ray microscopy.

Significance to the program of the Institute: The refinement of electron microscopy, both as regards attainable resolution of the instrument and delicacy of fixation and preservation of tissues, has given this laboratory high standing in the field. The pursuit of specific structural analyses of viruses and their states of development has resulted in laying the groundwork for study of fine structure and the relation of function to structure in other biological forms. The development of other techniques of visualization of fine structure and location and assessment of specific substances is opening new areas of study in microphysiology.

Proposed course of the Project: It is proposed that cooperative work on viruses with the Pasteur Institute be continued on a reciprocal basis of use of facilities and personnel and that the development of both electron and X-ray microscopy will be stressed in Bethesda.

Part B included Yes X

Part B.

Publications other than abstracts from this project:

Labaw, L. W., An electron microscopic determination of a tobacco virus crystal structure. J. Ultrastructure Research (In press).

Labaw, L. W. and Wyckoff, R. W. G., The electron microscopy of tobacco necrosis virus crystals. J. Ultrastructure Research (In press).

Mosley, V. M. and Wyckoff, R.W.G., Microradiography for absorption analysis. J. Ultrastructure Research 1: 337-348, 1958.

Wyckoff, R. W. G., Optical methods in cytology. Chapter in "The Cell". New York, The Academic Press (In press).

Serial No. 105
1. Physical Biology
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: The physical chemistry of membranes and complex membrane systems of biological interest.

Principal Investigator: Karl Sollner

Other Investigators: Rex Neihof, (left Jan. 5, 1958), Marc Lewis (left Jan. 14, 1958), Ruth McClintock, and Gerald M. Shean (since Oct. 2, 1958).

Cooperating Units: Loose cooperation is maintained with Dr. Charles W. Carr, Associate Professor, Department of Physiological Chemistry, Medical School, Univ. of Minnesota and with Dr. Eugene Grim, Associate Professor, Dept. of Physiology, University of Minnesota.

Man Years (calendar year 1958):

Total:	2-1/3
Professional:	2-1/3
Other:	0

Project Description:

Objectives: A physicochemical study of membranes and membrane model systems with the purpose of providing a rational physicochemical basis for the elucidation of numerous phenomena in living organisms, for instance, electrolyte balance and electrolyte distribution, the accumulation of electrolytes in living cells, cell and nerve potentials, and electrophysiology in general.

Methods Employed: The preparation of porous membranes of highly characteristic and specific electrochemical properties (the methods having been worked out by the principal investigator and his collaborators), and recently also of oil membranes of somewhat similar characteristics and the investigation of these membranes, and of membrane systems in which such membranes

Methods Employed (Cont'd):
are functional parts, by physicochemical, especially electrochemical methods, such as potential and resistance measurements, also by chemical analytical procedures, including radioactive tracer methods.

Major findings: Earlier theoretical considerations had led to the prediction that the ratios of the rates of the electrical transportation across permselective membranes of any two species of ions of the same charge coexisting in solution, should be predictable quantitatively, a) from the bi-ionic potentials arising with the same ions across the same membrane, and b) from the ratio of the rates of the exchange of the same two ions across the same membrane against a third ion. The experimental results were in fair agreement with the predictions. However, significant deviations, outside of the range of the experimental errors, occur regularly. We must conclude that, an electrophoretic interaction occurs between ions of the same charge when they are transported electrically across ionic membranes. At higher current densities the situation is still more complicated by polarization. The problem has turned out to be so complex as to make it advisable to terminate the work in this direction, at least for the time being.

Preliminary work on "oil" membranes indicates that this problem, which has been more or less dormant for many years, may now be taken up with some chance of success, by making use of more recently developed materials and techniques.

Significance to Research of the Institute: In order to understand electrolyte relationships in living cells and tissues, it is necessary to have accurate information on membrane model systems which, under carefully controlled known conditions, reproduce at least some of the major *in vivo* phenomena. The work of recent years, particularly the study of polyionic potentials, of absolute and relative rates of ionic fluxes under various conditions, and the construction of an *in vitro* model of electrolyte accumulation have brought us significantly nearer to an understanding and an *in vitro* reproduction of the type of effects which ultimately must govern the *in vivo* osmotic behavior of cells and tissues. The work already carried out indicates that even fairly complex membrane systems, similar to those found in living nature, may prove in the foreseeable future amenable to a complete and quantitative physicochemical analysis.

Proposed Course of Project: Finishing the current studies on the relative rates of the electrical transportation across permselective membranes of two coexisting species of ions of the same charge. Continuation of the theoretical and experimental work on electrolyte accumulation. Further work on membrane equilibria, particularly certain peculiarities of membrane equilibria in ionizing solvents such as water. Continuation of the work on absolute rates of exchange of ions across permselective membranes from the experimental and theoretical point of view. A systematic study of liquid, homogenous phase ("oil") membranes as a continuation of the work of Nernst-Haber-Beutner and Osterhout-Stanley-Shedlovsky et al., starting with the creation of membranes of high ionic selectivity, high ionic specificity and reasonably low conductance. A preliminary study concerning the forces involved in the formation of regular structures by means of the exploration of long range forces of attraction and repulsion between microscopic and macroscopic particles. (The basic experimental techniques have been worked out and tested by the senior investigator before coming to NIH.) These latter studies are designed to furnish an insight into the physical forces which create organized structures of various levels of complexity and size as those existing in living systems.

Part B included Yes

Serial No. 105

PHS-NIH
Individual Project Report
Calendar Year 1958

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Sollner, Karl: The Physical Chemistry of Ion Exchange Membranes. Svensk Kemisk Tidskrift 70, 267-295 (1958).

Lewis, Marc and Sollner, Karl: Preparation and Properties of Improved Protamine Collodion Matrix Membranes of Extreme Ionic Selectivity. J. Electrochem. Soc. (in press).

Serial No. 106
1. Physical Biology
2. Photobiology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: General Project Domain of Section - Molecular Mechanisms of Radiant Energy Transformation in Biological Structure.

Principal Investigator: F. S. Brackett

Other Investigators: Ralph G. Adams, William A. Hagins, Ulrich Weiss, Gerald R. Gauvin, Charles E. Lohr, Jr., Lawrence Showkeir
See projects: (a) Liddel, (b) Sharpless, (c) Becker, (d) Charney, (e) Olson

Cooperating Units: Laboratory of Technical Development, Heart Institute (see Serial No._____), Dept. of Terrestrial Magnetism of the Carnegie Institution, and Naval Medical Research Institute

Man Years (calendar 1958):
Total: 8
Professional: 4
Other: 4

Project Description:

Objectives:
Studies In Vivo
1. Action of radiant energy on structure and chemosynthesis in living cells. - Dr. Olson, Dr. Greenblatt, and Mrs. Engel.
2. Photochemistry and energy exchange in photoreceptors. - Dr. Hagins.
3. Relation of fluorescence and oxygen exchange to incident radiation. - Drs. Brackett, Olson, Greenblatt, et al.

Investigations of the action of radiant energy on biologically important molecules.
4. Photochemistry of steroids. - Dr. Sharpless, Mrs. Tenmer; Dr. Brackett, Dr. Weiss collaborating.
5. Photochemistry of organic molecules - particularly natural photodynamic pigment. - Dr. Weiss.

Spectroscopic investigations of related structures.

6. Infrared and NMR studies of hydrogen bonding. - Dr. Becker and Mr. Bradley. - Dr. Liddel collaborating.
7. Studies of the role of unpaired electrons (just beginning - will involve Dr. Adams).
8. Molecular scattering in relation to energy transforming structure. - Dr. Charney.

Instrumental development.

9. Appropriate innovation and adaptation of instruments to specific project needs. - Dr. Brackett. (Charles Lohr and Lawrence Showkeir)

Projects 1-8 constitute the major part of the closely related program of experimental investigation of this Section and will be discussed more fully by the project leaders. Less closely related is the new field being developed by Dr. Liddel in addition to his participation in our spectroscopic studies: See Project Title - Effect of nuclear radiation on biological systems.

Dr. Weiss, Dr. Hagins, and Dr. Adams have been with the Section so short a time that no separate report will be made on their projected studies.

Major Findings: (See separate projects for fuller discussion.)

1. Progress has been accomplished in relating the laminated structure in the chloroplasts to pigment distribution and fluorescence. Partial evidence of the role of these structures in the photochemistry has been found.
2. Just initiated.
3. Instrumentation nearing completion.
4. Using a chemical endpoint the quantum requirement for ergosterol transformation to other isomers was determined and found to confirm our earlier unpublished work based entirely on spectral evidence- 2.6-2.8 quanta per molecule at 2650A. Work is being extended to other wavelengths.
5. Dr. Weiss has contributed greatly to our steroid work but has not had his laboratory yet because of PHS delays.
6. Nuclear magnetic resonance data have been obtained to establish essential reference bases - unspectacular but necessary for good work. Hydrogen bonding has been shown to impose intensity changes in the H-C vibrational frequencies in the infrared.
7. Dr. Adams has just reported for duty.
8. The relation of algal light scattering to the anomalous dispersion by chlorophyll has been confirmed and shown to have its seat within the chloroplast.

The essential nature of protein binding has been demonstrated by enzyme digestion (Proj. 1) and independently shown in model experiments with porphyrins and proteins (Proj. 8).

9. Original instrumentation continues to play an important part in the research.

a) A double monochrometer comprising grating and fused quartz components is nearing completion. Evaluation of spectral properties either through an integrating sphere or microscopic attachments is provided. Components may be rearranged for studies of fluorescence or scattering.

b) A grating system is also nearly completed for studying quantitatively the simultaneous action of two or more monochromatic contributions to photo action. Provision is made for crossfiring by two incident beams producing homogeneous light absorption and also for evaluation of transmission by integrating sphere.

c) Proposals for the constructing of a digital logging system are being presented. This will be the first step in the greater use of digital computers for spectroscopic analysis.

d) An analogue instrument for studying the composition of complex spectral absorption is being assembled from parts constructed by the NIH Electronics Shop. The design is by Dr. Murray Eden and Dr. Frank Noble of the Laboratory of Technical Development, Neart Institute.

Significance to Research of the Institute: Radiation supplies not only the energy required by living things but also the most essential information which largely determines their activities. These studies are designed to contribute to our basic understanding of these mechanisms.

Proposed Course of Project: Our researches are just reaching productive maturity with a well-balanced staff on the one hand and several instrumental developments being completed on the other hand. A marginal amount of space becomes available by completion of the west areaway, Bldg. 2. Members of the staff are working at the Naval Medical Research Institute and at the Department of Terrestrial Magnetism of the Carnegie Institution, as well as in Building 10.

Part B included: Yes

Serial No. 106

PHS-NIH
Individual Project Report
Calendar Year 1958

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Brackett, F. S. and Hollaender, A.: Remarks on the significance of action spectra. Report on Conf. on Photomorphogenesis held at Gatlinburg, Tenn., Oct. 1957 (in press).

Brackett, F. S.: Book review of "Physical Optics", R. A. Houstoun, Interscience Publishers, New York. Rev. Scientific Instruments (in press).

Serial No. 107
1. Physical Biology
2. Photobiology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Molecular Structure and Organization in Biologically Important Systems.

Principal Investigator: Elliot Charney

Other Investigator: Susan Rupp (summer employee)

Cooperating Units: None

Man Years (calendar year 1958):
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: The general objectives of this research are to explore the nature of intermolecular interactions, the role they play in molecular organization and the relation of this organization to biologic activity.

Methods Employed: Several investigations have been undertaken: one of these involves measuring the spectral dependence of scattering from single cell algae. Another is the investigation of the structure of molecular complexes by solution spectroscopy. The investigation of the solid state chlorophyll lipoprotein complex started last year has been at least temporarily set aside in view of the retraction by Takashima (Arch. Biochem. and Biophys. 74, 478 (1958)) of his earlier reported crystallization of this complex.

Major Findings: The experimental determination of the spectral dependence of scattering from viable chlorella pyredenosae and its dependence on the refractive index of the surrounding medium and the size of the chlorella (the diameter of this single celled algae can be varied over a five-fold range by controlling the growth conditions) has been completed. The results, which have

been partially analyzed and are being prepared for publication confirm the reported anomalous scattering near absorption bands. A theoretical analysis based on the rapid fluctuation of the refractive index of the chlorophyll pigments in absorbing regions in these algae gives remarkably good agreement with the observed data considering the gross nature of the structures in these living cells. That closeness of the agreement demonstrates that the pigment plays an important role in the scattering. Additional evidence for this is obtained from the data which indicates that relatively only a small portion of the scattering has its origin at the cell wall, so that the reduction of total scattering by suspending the cells in a medium of apparent refractive index match does not suffice to eliminate pronounced scattering in an absorption band. Measurement of the refractive index of the chloroplast in these cells by phase microscopy indicates that the relatively large scattering contribution from this inner particulate undoubtedly arises not only from the fluctuation of the refractive index but also from the magnitude of the index difference relative to the surrounding protoplasm resulting from the high density of this chlorophyll lipoprotein structure.

The absorption intensity of the Soret peak of metalloporphyrins is a sensitive indicator of intermolecular interactions. Dilute aqueous solutions of, for example, copper chlorophyllide in admixture with other hydrogen donor solvents show a marked increase in the peak and integrated intensities of the Soret band at about 405 mμ, with decreasing water concentration. The behavior in dilute protein solutions has both marked similarities and differences, the increase in peak intensities apparently being accompanied by a slight decrease in the integrated intensity. The intensity changes with bovine serum albumin indicate a specific binding behavior with small mol ratio. We are presently studying the binding behavior as a model with respect to its possible implications in the gross structure of intact biological systems.

Significance of the Program to the Institute: It has long been recognized that sub-microscopic (molecular) organization is the basis for much of the structure of living organisms. The role organized molecular structures play in biologic activity in general and in energy transfer in particular is only partly elucidated. Using the probe of electromagnetic radiation with biologically active chromophores such as porphyrin pigments or more

simple analogues, we hope to make further advances in a fundamental understanding of these phenomena.

Proposed Course of Project: The porphyrin solution spectroscopy will be continued along at least two lines. One is the general nature of molecular interactions as determined by intensity changes and spectral shifts of the electronic and vibrational transitions of these molecules and the second is the specific nature of the binding between porphyrins and proteins. If, as is indicated by the results to date, this binding permits us to study the behavior of the binding sites during protein denaturation, and possibly even during enzymatic activity, this line of investigation will be pursued.

Two other investigations are in early stages of planning. These involve again the determination of molecular structure and molecular interactions but in this case by examining the effect of strong electric fields on the spectra of the absorbing systems. In one of these we hope to help elucidate the origin of the dipole moment of the charge symmetric molecule p-benzoquinone and in the other examine electric-birefringence in proteins. The latter is based on the role which the molecular structure plays in determining the anomalous deviation of birefringence in infrared absorption bands (Charney and Halford, Jour. Chem. Phys. 26, 221 (1958)).

Finally, in collaboration with Dr. Ulrich Weiss, we are preparing to examine the possibility of measuring optical rotatory dispersion in the infrared region of the spectrum.

Part B included Yes No x

Serial No. 108
1. Physical Biology
2. Photobiology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Molecular structure determined by spectroscopic methods

Principal Investigator: Edwin D. Becker

Other Investigators: Robert B. Bradley

Cooperating Units: None

Man Years (Calendar year 1958):
Total: 2
Professional: 2
Other: 0

Project Description:

Objectives: (1) An understanding of the forces within and between molecules, especially those of potential biological importance. (2) Development of spectroscopic methods for studying molecular structure and analyzing for materials of biological interest.

Methods Employed: The primary techniques used in this work are infrared spectroscopy (IR), nuclear magnetic resonance (NMR), and electron paramagnetic resonance (EPR). The IR and NMR methods supplement each other in providing detailed information about the structure of molecules. In addition, both types of spectra are highly sensitive to the effects of molecular interactions and are used to study such interactions. EPR is used in studies of molecules having unpaired electrons, including free radicals and paramagnetic atoms and ions.

Major findings: The project can be divided roughly into two parts: (a) studies of hydrogen bonding and other molecular interactions, utilizing both IR and NMR; (b) more general investigations of molecular structure by NMR and EPR.

(a) We have made quantitative investigations of some of the more subtle effects of hydrogen bonding on the IR spectrum, particularly changes in the intensity of absorption bands due to C-H vibrations in alcohols and in chloroform. Our IR spectrometer has been substantially improved by the addition of diffraction gratings to provide better resolution and a logarithmic potentiometer to permit direct recording of optical density. These modifications have improved the accuracy of the spectral measurements and will speed the processing of data.

The effect of molecular interactions on compounds used as reference substances in NMR studies has been investigated as a necessary prelude to a more extensive study of hydrogen bonding by this technique. Tetramethylsilane and cyclohexane appear to be satisfactory reference materials for such studies.

(b) We have conducted exploratory work with NMR in investigating a wide range of compounds. In addition to substances from our own laboratory, ranging from amino acids to steroids, compounds have been provided by other investigators at NIH. By means of these studies we are developing useful correlations and establishing the scope of applicability of the technique. Similar preliminary investigations have been conducted with EPR. The recent acquisition of additional equipment is expected to accelerate this phase of research.

Significance to Research of the Institute: The further development of NMR and EPR is expected to be of considerable assistance to many NIAMD scientists, since these methods will add two more spectroscopic techniques that can be brought to bear on biological problems. For example, NMR spectra of steroids and other complex molecules can be helpful in unraveling their molecular structure. EPR studies may permit the detection of free radical or paramagnetic intermediates in reactions of biological significance.

A deeper understanding of the properties of hydrogen bonds is clearly desirable since such bonds are of prime importance in determining the structure and function of proteins and nucleic acids.

Proposed Course of Project: (1) We plan to continue the studies previously reported on hydrogen bonding in an attempt to determine and correlate thermodynamic and spectroscopic properties of hydrogen bonds in various systems. (2) The installation of recently purchased accessories for our NMR equipment will materially increase our capability of studying complex molecules. We expect to focus our NMR structural work on several classes of compounds, including long chain fatty acids, steroids, and, if possible, porphyrins. (3) By means of collaborations with other investigators in NIAMD we shall attempt to use EPR to examine the role of free radicals in suitable biological systems.

Part B included: Yes

PHS-NIH
Individual Project Report
Calendar Year 1958

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Becker, Edwin D., Liddel, Urner, and Shoolery, James N.: Nuclear magnetic resonance studies of hydrogen bonding in ethanol. J. Molecular Spectroscopy, 2: (1) 1-8, Feb. 1958.

Serial No. 109
1. Physical Biology
2. Bionucleonics
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Effect of Nuclear Radiation on Biological Systems

Principal Investigator: Urner Liddel

Other Investigators: Frank M. DeFilippes, Ellis S. Kempner

Cooperating Units: Department of Terrestrial Magnetism, Carnegie Institution

Man Years (calendar year 1958):
Total: 3
Professional: 3
Other: 0

Project Description:

Objectives: (1) An understanding of the effects of neutrons and X-radiation on biological systems (a) by study of the molecular interactions which occur as a result of these radiations (b) by study of cytological changes which occur under radiation.

Methods Employed: Radiation of cultures of micro-organisms and subsequent examination by microscope and other physical methods - e.g., induced radioactivity, spectroscopic analysis where feasible, including absorption and electron paramagnetic resonance.

Major Activities: Space for research on the effects of nuclear radiation in biological systems was obtained, on loan, in the late spring. Modifications for use of part of this space were drafted by Plant Management and released for bid in the month of December.

Two Ph.Ds in Biophysics are now members of the staff and beginning to initiate experiments as equipment and work areas become available. One is working in the facilities of the Department of Terrestrial Magnetism, through the courtesy of the Director of that Department, on the amino acid sequence in protein.

A source of neutrons should be delivered early next

year, and if the laboratory area is in usable condition experiments on the effects of neutrons should be started. A 200 kv X-ray machine, perhaps in working condition, is also available for X-radiation studies.

Other personnel to increase the activity in this area are under active recruitment and await Civil Service certification.

Significance to the Program of the Institute: This activity is a part of the general biophysics study of the mechanisms of energy transfer in molecules. High energy radiation is absorbed and degraded by various means. Knowledge of these means will give us a better understanding of the forces effective in molecules of biological importance. From a general health standpoin a knowledge of the effects of low energy neutrons is essential.

Proposed Course of Project: In addition to general studies mentioned above, more specific studies of the photo-reactivation of radiation damaged cells; studies of the amino acid sequence in proteins; on the effect of neutrons on single cells or simple organs such as the lens of the eye, under tissue culture; and the effects of radiation on the biological rhythms exhibited by many simple organisms.

Although we have not yet been able to devise the proper adjacent location of apparatus, we plan to explore by physical means the formation of free radicals in radiated systems, including various regions of the electromagnetic spectrum.

Part B included Yes x No

Serial No. 109

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Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Liddel, Urner and Becker, Edwin D.: Infrared spectroscopic studies of hydrogen bonding in methanol, ethanol and t-butanol. Spectrochimica Acta, 10: 70-84, 1957.

Serial No. 110
1. Physical Biology
2. Photobiology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Investigations of the Action of Radiant Energy on Biologically Important Compounds

Principal Investigator: Norman E. Sharpless

Other Investigators: Odette S. Yemmer

Cooperating Units: None

Man Years (calendar year 1958):
Total: 1-3/4
Professional: 1-3/4
Other: 0

Project Description:

Objectives: The objectives of this project are the establishment of various biologically important intermediates in photobiology and to study their kinetics and other physical and chemical properties.

Methods Employed: Ultraviolet or visible radiation is the means employed to effect any alterations in the materials under investigation, and spectroscopy in the ultraviolet, visible and infrared regions is the major method used to evaluate any changes which might occur.

Major Findings: The photochemical conversion of ergosterol and related steroids into the various vitamins D is a biological photoreaction of the utmost importance, ranking with photosynthesis and vision.

The mechanisms and quantum efficiencies of these reactions are still unknown, the structures of some of the products are in doubt, and even the existence of some previously reported products is now in dispute.

The photochemical change of ergosterol is complicated by the photosensitivity of the intermediates formed, by their similar spectra, and by the similarity of the intermediate resulting in difficult chemical separations.

This one method which is now being refined, is the quantitative estimation of the residual ergosterol by precipitation with digitonine. By evaluation of such ergosterol as a function of absorbed radiation and extrapolation to zero radiation to correct for the competition of the other products it has been established provisionally that the disappearance of ergosterol from an ethanolic solution requires about 2.7 quanta per molecule at 2660 A. A method for the quantitative estimation of tachysterol, an important photoisomer of ergosterol, is being developed.

It is well known that changes in the side chains of the provitamins D have no sensible effect on the course of photochemical change, as long as the 5,7 double bond system of the nucleus is unaffected. However, less is known regarding the effects of changes in the rings or ring substituents. A partial investigation of these effects has been qualitatively carried out, using thioergosterol (5,7,22 ergostatriene-3(β)-thiol) where the hydroxyl group has been changed, and 5,7 estradiene-3β,β17 diol. Both of these compounds were irradiated with λ2537 A. The thio compound showed changes strictly analogous to those of ergosterol, indicating that replacement of oxygen with sulfur had no qualitative effect on the course of the photochemical changes.

Irradiation of the estradiene diol was qualitatively different. This substance is a 19 nor compound compared to ergosterol, and therefore cannot form a compound analogous to tachysterol. The spectral results bore this out.

The effects of various metabolic inhibitors on the formation of phosphytin in Euglena gracilis were studied kinetically as a function of pH and inhibitor concentration. The extent of pigment changes was dependent on (in one series of experiments) the concentration of 2,4 dinitrophenol and external pH. The dinitrophenol produced more drastic changes in pigment than acid alone. The results so obtained were

been discussed in relation to the intracellular accumulation of hydrogen ions, in a paper submitted to the Journal of Protozoology (Greenblatt, C. L. and M. E. Sharpless, "Effects of Some Metabolic Inhibitors on the Pigments of Euglena gracilis in an Acid Medium.

Significance to the Program of the Institute: A proper understanding of the behavior of biologically important molecules on a molecular level is absolutely necessary to the extrapolation of their effects to a cellular level for the evaluation of their roles in health and disease.

Proposed Course of Project: It is proposed to continue the quantum yield of ergosterol disappearance as functions of wavelength, concentration and temperature, as well as the other products. Further work on the direct determination of tachysterol is in progress and will be continued. The isolation and structural studies of toxisterol will be carried out in collaboration with Dr. Ulrich Weiss of this laboratory, and further work and structurally similar compounds such as thiosterol will be attempted. The use of radioactive ergosterol in these studies shows great promise and may be the only tool available for the quantitative evaluation of such products as lumisterol and suprasterol.

Part B included Yes No x

Serial No. 111
1. Physical Biology
2. Photobiology
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: The action of radiant energy on structure and chemosynthesis in living cells

Principal Investigator: Rodney A. Olson

Other Investigators: Charles L. Greenblatt and Estella K. Engel

Cooperating Units: None

Man Years (calendar year 1958):
Total: 3
Professional: 2
Other: 1

Project Description:

Objectives: The study of photochemistry at its cytological site, the elucidation of mechanisms of energy transfer involved, and an understanding of the influence of microstructural environment on such processes.

Methods Employed: Problems in three areas of methodology related to our work were described in the previous annual report. These included the uniform culture of the photoactive unicellular organisms Chlorella and Euglena, the rapid electrolytic measurement of their oxygen and CO_2 metabolism in the presence of interfering specific inhibitors, and the absorption and fluorescence microscopy of their photoreceptor systems. Current methods employed in this direction and their contribution to the solution of these problems are as follows: Culture variation has been chiefly eliminated from a morphological standpoint by synchronous growth via intermittent light and dark period "phasing". Metabolic rate measurement by an electrolytic method in the presence of disturbing ions was made possible

by a hydrophic membrane-flow electrode developed for the rapid simultaneous determination of CO_2 and O_2. Perfection and application of this method has been postponed, however, in favor of more immediate requirements of specialized absorption and fluorescence microscopy. We have therefore emphasized the study of photochemical sites of action and associated features of molecular architecture. Previous photodensitometric representation of absorption and fluorescence change has been replaced by the more rapid and direct quantitative radiometric approach. The present use of interference filters to obtain simultaneous measurements at selected wavelengths is employed as a compromise awaiting the development within the section of a more refined optical system in which the whole spectrum may be rapidly scanned. Such a cooperative project is planned in the near future (see Dr. Brackett's report).

Major Findings: In an attempt to correlate morphological with functional aspects of radiant energy utilization we have carried out further studies of chloroplast structure. Absorption microscopy as a monitor of pigment distribution in swelling and enzymatically treated chloroplasts reveals information which correlates with electron microscopy of the same material. These studies strongly support our previous concepts of the submicroscopic structure of chlorophyll lamellae *in vivo*.

In functional studies of the character and kinetics of photochemical reactions at lamellar sites, the bleaching of chloroplast pigments has been followed. The usual red fluorescence prior to this bleaching is replaced by a greenish-white emission. This profound alteration in the wavelength of emission and absorption is partially reversible and is altered by the physiological state of the organisms. These changes recorded simultaneously at selected wavelengths, present data characteristic of pigment participation in the photochemical reaction.

Metabolic studies have been carried out in the maintenance of chloroplast pigments in *Euglena*. These pointed to energy expenditure for maintenance of ion transfer in *Euglena* in acid medium. Morphological studies of the distribution of chloroplast pigments were correlated with pigment absorption and the influx of acid into the organism.

Significance to the Program of the Institute and Proposed Course of Project: Unconventional biological application of such optical and electrochemical instrumentation provides insight into processes of energy transfer which occur in the specialized systems under study. This may be applied to all living cells whether light or chemically activated.

Future studies of emission and absorption spectral changes at their cytological sites may elucidate other photochemical mechanisms and allied metabolic changes. Correlation of metabolic and photochemical behavior will require further adaptation of electrode techniques. Finally, we hope to further refine these methods for application to cellular reactions of a more general occurrence.

Part B included Yes x No

PHS-NIH
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Calendar Year 1958

Part B:

Publications other than abstracts from this project:

Greenblatt, C. C., and Schiff, J. A.: The occurrence of a pheophytin-like pigment during dark adaptation of Euglena gracilis var. bacillaris. J. Protozoology, in press.

Olson, R. A., and Engel, E. K.: "Chlorophyll" absorption microscopy of in vivo, cell free, and fragmented Chlorella chloroplasts. Brookhaven Symposium on the Photochemical Apparatus, in press.

Olson, R. A., and Engel, E. K.: Visible absorption microscopy of pigment systems in living cells using interference filters: Chlorella chloroplast. Proc. Microscopy Symposium, Chicago 1958, Interscience Publishers, in press.

Honors and Awards relating to this project:

None

PHS-NIH
Individual Project Report
Calendar Year 1958

Serial No. NIAMD 112
1. OADR
2. Mathematical Research
3. Bethesda, Maryland

Part A.

Project Title: Mathematics of kinetics and reaction-transport systems.

Principal Investigator: John Z. Hearon

Man Years:
Total: 1 1/2
Professional: 1
Other: 1/2

Objective and Method of Procedure: The chief objective is to conduct a systematic study of the mathematical problems of complex reaction systems (e.g., catalytic systems involving; cofactors, modifiers, inhibitors, etc.) and systems in which chemical conversion and translocation of matter simultaneously occur (e.g., intact cells and/or organs, flow systems, open systems in general). This project is expected to produce solutions of specific problems and to provide predictive models suggesting further and perhaps novel experimentation. But it is held to be equally important to deduce general properties, to contribute to sound concepts and "philosophy" and to establish limitations set by general physical and mathematical principles (see examples under Major Results). This project requires a "parallel approach" in that several basic problems, and their relevant areas of mathematics, are carried forward simultaneously, (e.g., homogeneous enzyme kinetics and the diffusion problems are developed side by side, ultimately to be synthesized; the rate behavior of a system is ultimately governed by the thermodynamics of the system thus certain aspects of kinetics and energetics must be carried forward together). The emphasis is quite frankly shifted from time to time in response to the demands of application, and as a result consultation with those seeking aid and advice from the Office of Mathematical Research. Further there are often corrolaries of mathematical results which have application in other subject-matter areas.

Major Results and Significance:

Steady-state Kinetics; In continuation of work reported for calendar year 1957, a rather complete formulation of the temperature-dependence of the steady-state rate has been achieved. It has been shown that the thermodynamic nature of the temperature coefficient (traditionally and incorrectly interpreted in terms of the Arrhenius equation) is determined by whether the catalytic species are performed

...n the system or part be closed by equilibrium steps certain ... (as the formation of hexokinase from apo- and co-enzyme) ... for rate limiting steps in various types of catalytic ... have been established. In particular it has been shown that a linear Arrhenius plot does not imply the existence of a rate limiting step nor can the slope of this plot necessarily be identified as an energy of activation. The conceptual and practical importance of these results is evident.

Matrix theorems and linear analysis: It has previously been shown by this investigator that the rate matrix for a closed linear (chemical or transport) system is necessarily similar to a symmetric matrix. This mathematical result is implied by the physical principle of detailed balance. The counterpart of this theorem has now been proved for an arbitrary chemical system. The consequences, and the required proof of new results in the realm of similarity and symmetric relations among matrices, are almost innumerable. A few are: No linear system (open or closed) and no closed system can exhibit oscillatory or periodic behavior; there must only certain ways of opening an arbitrary closed system which lead to the possibility of oscillatory behavior and such behavior must of necessity be sought among a well-defined class of open systems. Exploitation of certain symmetry properties permit the estimation of equilibrium constants, free energies and heats of reaction from studies of rates alone and in systems which do not come to equilibrium (and thus are not susceptible to classical mass action study). The complete solution of "relaxation times" for non-linear systems is now available and the principle can furnish on the basis of routine chemical analysis information which otherwise can be obtained only by tracer studies. Certain fitting and computational problems are greatly simplified and in particular choice of computer methodology is dictated at once by the analytical results.

Kinetics of Intact Cells: A formulation is available which is restricted to first order or zero order kinetics. A very simple approximate version is available when the ratio of cell volume to total volume is appropriately restricted. Given this volume ratio an upper limit for the error of approximation can be stated. This error analysis involves new theorems about homogeneous functions (of mathematical interest per se) and is applicable to certain other problems (see 1 and 2 under collaboration). These results show the error of ones ways when cellular reactions are considered within the framework of homogeneous kinetics and exhibit conditions under which agreement with homogeneous kinetics can be secured. In addition practical answers are provided to these questions: Does a given inhibitor affect permeability, enzyme activity or both? Are the same parameters, say turnover number, of a given enzyme the same in vivo and in the isolated purified state? Can one infer cellular concentrations from analysis of the medium? In addition the analysis provides estimation procedures for permeability coefficients of metabolites

(let it be noted here that such coefficients are available by and large for the most uninteresting and non-physiological compounds because the fact that a substance is consumed by the cell is a great complication in the usual methods of estimation).

An Area Theorem: There are many problems which require knowledge of the integral

$$\int_0^\infty f(t)dt,$$

where f(t) obeys a certain differential equation, and it is demanded to know the value of the integral (area under the curve) in terms of the parameters of the differential equation. This problem has been solved in general for a set of n such functions obeying n simultaneous linear differential equations. The method, purely algebraic and without recourse to the solutions of the differential equations, finds application in estimation of radiation dose to an organ; estimation of migratory aptitude in the pinacol rearrangement; final yield of thrombin from prothrombin, in the presence of a clotting inhibitor.

Collaboration and Consultation: This investigator has had queries from and discussions with members of every Institute except NIDR. These have ranged from critical analysis to actual problem solving. The following are examples from which joint publication will result or in which an "analytical memo" has been prepared in behalf of the consultee.

1. Dr. S. Wollman, NCI-LPBI. Asymptotic behavior of ratio of thyroid curve (radioiodine) to blood curve and conditions under which "lumping" occurs between two compartments for an arbitrary blood curve.

2. Drs. D. Stetten and F. Tietze, NIAMD-LBM and Dr. C. Mortimore, NIAMD-CE. Kinetics of perfused organ system. Time course in reservoir of metabolite consumed in organ.

3. Dr. N. R. Shulman, NIAMD-MD. Analysis of kinetics of thrombin formation in presence of inhibitor.

Proposed Course: It is proposed to continue certain purely mathematical investigations in the realm of matrix calculus. There are extensive plans to study the effect of the spatial distribution of a catalyst on reaction rate with special reference to the structured nature of enzymes within cells. Some of the analytical work has been done but much computational work remains to be done. There is also planned extensive computation in connection with assessing approximation methods for solving the equation of continuity, in particular multi-region diffusion-reaction problems.

Part B. included.

Part B:

Publications:

Mesrob, J. S., Bernhard, S. A., Friess, S. L., Botts, J. and Morales, M. F. Enzyme Kinetics, Chapter in The Enzymes, (ed. 2), edited by Lardy, Boyer and Myrback. New York, Academic Press, Nov. 1958.

Best, J. B. and Mesrob, J. S. Thermodynamics of Homeostasis, Chapter in Mineral Metabolism, edited by Bronner and Comar. New York, Academic Press. In press.

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Individual Project Report
Calendar Year 1958

Serial No. NIAMD 113
1. OADR
2. Mathematical Research
3. Bethesda, Maryland

Part A

Project Title: Analysis of Radioisotope Tracer Data

Principal Investigator: Mones Berman

Cooperating Units: Sloan Kettering Institute, New York, N. Y.
New York Hospital, New York, N. Y.
Clinical Endocrinology Branch, NIAMD
Serial No. 135-C

Man Years:

Total	1 1/2
Professional:	1/2
Other:	1

Project Description:

Much of the work reported here was done at the Sloan Kettering Institute, N.Y.C., N.Y. This investigator started in his present position on June 12, 1958.

Objective: The objective of this project is to develop a mathematical and computational methodology for the systematic and routine interpretation of tracer data on steady state biological systems. The methods under development are intended to provide a rationale for how to choose a physiological model for a set of data and how to treat the data for the model chosen. It is also intended to program the procedures for routine use on analog and/or digital computers, and make them available to other investigators.

Methods Employed:

1) Mathematical theory: The development of a theoretical basis for the procedures of analysis to be used.

2) Analog computer: The construction, testing and solution of special models for which direct mathematical analysis are not practical. Methods are being considered for obtaining a model and its solution in a systematic manner.

3) Digital computer: The programming of already developed methods on a digital computer for routine and speedy solutions.

Major Findings:

1) Quantification of systematic and statistical uncertainties in the data that permits the association of a figure of confidence to the values of the parameters of the physiological models.

2) The programming of part of the developed procedures on a high speed digital computer (IBM 704) for routine use. The program permits the fitting of data to a sum of exponentials using a modified least squares procedure and obtains the statistical uncertainty for the constants of the fit.

Significance to the Program of the Institute: A great deal of work is carried on using isotope tracer techniques. Interpretation of the collected data is usually made by postulating some compartmental model and solving for turn-over rates and compartment sizes of the model. It is assumed that the values obtained may either reflect mechanisms of action or indicate the sites of normal and abnormal processes. Such an analysis of data is limited at present to very simple systems because of the complexity in the analysis of multi-compartmental systems. It is hoped that the methods developed here will enable investigators to study more complex systems, extract more information from their data and have a measure of confidence for the models they propose.

Proposed Course of Project: The course of the project will continue along the same lines outlined earlier. The development of mathematics for rigorous procedures in formulating biological models and in analysing data will continue. Whenever any phase of this is completed to a point where it will be useful to put on a digital computer--it will be programmed and made available for routine use. The development of the use of the analog computer will continue whenever the mathematical procedures are either impractical or do not yield the kind of insight desired.

Part B not included

PHS-NIH
Individual Project Report
Calendar Year 1958

Serial No. NIAMD 214
1. OADR
2. Mathematical Research
3. Bethesda, Maryland

Part A.

Project Title: Study of iodine kinetics in the thyroid-system and radioiodine treatment of thyroid abnormalities.

Principle Investigator: Mones Berman

Cooperating Units: Sloan Kettering Institute, New York, N.Y
New York Hospital, New York, N.Y.
Clinical Endocrinology Branch, NIAMD,
Serial. No. 135-C

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Much of the work described here was done at the Sloan Kettering Institute, New York, N.Y. This investigator started in his present position on June 12, 1958.

Objective: The objective of this project is to develop a general model for the kinetics of iodine in the thyroid system that will account for the various thyroid disorders found in patients. It is also the purpose of this project to learn about the sites of action of radioiodine when used for therapy and the effects on iodine turn-over rates produced by radiation and drugs.

Methods Employed: The studies are performed on patients with various thyroid abnormalities. Measurements are made on the radioactivity in the thyroid, blood, PBI and urine over a period of several weeks after a single tracer injection. The turn-over rates of the various compartments are then determined from the collected data. These studies are done on the same patients before and after various changes are induced by radiation treatment or administration of chemical agents. Two radioisotopes of iodine have been employed for radiation treatment: I-131 (8d half life) and I-133 (21 hr. half life).

Major Findings:

1) Clinically defined hyperthyroid patients differ in the nature of their iodine kinetic patterns to suggest more than a single class of hyperthyroidism.

2) Radiation and chemical agents do not necessarily affect the same mechanism in all hyperthyroid patients; nor do they affect the same mechanisms in the same patient at various stages of abnormality.

3) The conventional model proposed for iodine metabolism is inadequate to explain some iodine kinetic patterns obtained on abnormal thyroid patients.

Significance of Program to the Institute: Since the thyroid is a most important organ in the regulation of physiological processes, its detailed modes of action are of interest for understanding the thyroid as well as other metabolic systems in the body. Furthermore, the development of an analytical procedure to treat the homeostatic mechanism of the thyroid may also be applicable to other homeostatic mechanisms in the body.

Proposed Course of Project: Further studies will be conducted on patients with normal and abnormal thyroid physiology. This work will be done in collaboration with Drs. C. C. Isaacson and J. B. Hill of the Clinical Endocrinology Branch of the NIAMD. General models to account for all the experimental data collected will be formulated and tested using an analog computer. An effort will be made to correlate thyroid abnormalities and radiation treatment schedules with specific mechanisms in the iodine metabolism cycle.

Form B not included

Serial No. NIAMD A15

Part A

Project Title: Mathematical formulation and analysis of problems relevant to neurophysiology.

Principal Investigator: Wilfrid Rall

Man Years:

Total:	1
Professional:	3/4
Other:	1/4

Project Description:

Objectives: In general, the objective is to re-examine various qualitative theoretical hypotheses of neurophysiology and to develop explicit mathematical formulations which permit precise predictions and critical experimental testing.

Three different types of problems have been considered:

1) Spread of membrane potential disturbances over the soma-dendritic surfaces of a neuron;

2) External field of electric potential due to flow of electric current generated by a neuron;

3) The probability basis for the relation between the responses of individual neurons and populations of neurons.

Methods employed: An important preliminary to mathematical formulation is the definition of a theoretical model of the physiological system under study. Although such a model is usually simplified, it is important that it should include the most essential characteristics of the physiological system (therein lies the art of the method).

Problems of type (1) involve solution of a partial differential equation for a variety of boundary conditions and initial conditions. Some cases can be solved by means of Laplace transforms; others require infinite series solutions involving special attention to orthogonality properties.

Problems of type (2) involve the use of spherical harmonic solutions of Laplace's Equation with suitable singularities at sources and sinks of current flow. The coefficients of the infinite series must satisfy continuity of current across the nerve membrane.

Problems of type (3) involve the formulation of appropriate probability models, with special attention to covariance.

In all cases, an important objective is to devise methods of estimating the fundamental parameters of the theoretical model from the quantities that are experimentally measurable.

Major Findings and Significance:

(1) Successful solutions for the spread of membrane potential over tapering and branching dendritic systems have been obtained for a variety of boundary conditions and initial conditions. These results are relevant to recent experiments with intracellular microelectrodes; such measurements of neuron resistance and of membrane potential transients cannot be correctly interpreted without these theoretical results. The mathematical results are also relevant to theoretical hypotheses of synaptic excitation in the central nervous system.

It is significant that these results give the dendrites much more importance than neurophysiologists previously allowed. Anatomically, the dendrites have always been a prominent feature, but neurophysiologists have tended to slight their importance, mainly because dendrites could not be easily fitted into their theories. These mathematical results therefore provide a necessary bridge between neurophysiological theory and anatomical fact.

(2) Mathematically rigorous external field solutions have now been obtained for several special cases of particular interest, and other special cases can be generated from these. Full exploitation of these theoretical results will require extensive calculations, probably with a digital computer.

Such field solutions are important for interpretation of the large body of extra-cellular recordings made with microelectrodes in spinal cord and in cerebral cortex.

(3) It has been found that reflex excitability fluctuations (in cat spinal cord) can be analyzed in terms of a mathematical model which combines independent fluctuations inherent in individual neurons with correlated fluctuations due to a common influence, such as inter-neuronal activity in the spinal cord. The mathematical model will be reported (in an invited paper) to a joint meeting of The Biometric Society and The American Statistical Association. Because the central nervous system clearly involves both deterministic and stochastic processes, it is important to develop methods and models which fit the randomness and constraint observable in various sub-systems.

Proposed Course: Further work is intended for each of the three types of problems described. The basic theoretical results have already been obtained, but there is still much to be done in the application of these general results to various specific experimental situations. The selection of special cases for detailed application will depend to some extent upon the consultations being maintained with experimental neurophysiologists.

One rather comprehensive project, which would depend upon extensive use of the digital computer, would combine the theoretical results obtained for dendrites with the general solution obtained for the electric field external to a neuron. This would bring these theoretical results much closer to the extracellular recordings obtained by neurophysiologists.

Part B not included

Individual Project Report
Calendar Year 1958

Serial No. NIAMD 116
1. OADR
2. Mathematical Research
3. Bethesda, Maryland

Part A.

Project Title: Theoretical consultation with experimental neurophysiologists.

Principal Consultant: Wilfrid Rall

Investigators consulted with:

Dr. Karl Frank, NINDB-LNP
Dr. W. H. Freygang, Jr., NINDB-LNP
Dr. M. G. F. Fuortes - NINDB-O
Dr. J. del Castillo - NINDB-EEG
Dr. F. J. Brinley, Jr., - NINDB-LNP

Man Years:

Total: 1/4
Professional: 1/4
Other:

Project Description:

This is not a typical project; it is a collection of separate consultations with experimental neurophysiologists.

Objectives: The main objective is to apply mathematical results obtained with theoretical models of neurophysiological systems to the analysis and interpretation of experimental measurements being obtained by a variety of recently developed neurophysiological techniques.

Methods: Important aspects of the method are the following.

1. Critical discussion and analysis of the experimental situation to determine the essential characteristics which must be included in a satisfactory theoretical model of the system under study.

2. Mathematical treatment of this theoretical model, and perhaps also of the larger class of theoretical models which includes this one as a special case.

3. Special attention to the prediction of relations between various experimentally measurable quantities. This includes also recommendations for the best methods of plotting and analysing experimental results.

4. Estimation of fundamental physical parameters of the system from the experimental information that is available.

5. Recommendations for further experiments designed to provide experimental information necessary for more accurate and/or more complete interpretation of the neurophysiological system under study.

Specific Consultations and Findings:

(1) Consulted with Drs. Karl Frank and W. H. Freygang, Jr. (NINDB-LNP) on the underlying theory and the interpretation of extracellular potentials recorded with microelectrodes touching the surface membrane of a motoneuron. (Joint abstract in Fed. Proc. 1958; their paper submitted to J. Gen. Physiol.). Theoretical calculations, together with observations made on an electrical analog constructed by Mr. A. J. McAlister at the Naval Medical Research Institute, pointed to the possible role of nerve membrane "dimpling" beneath the electrode tip. A major result was confirmation of the hypothesis that at least part of the neuron soma membrane does not generate a nerve impulse.

(2) Consulted with Dr. M. G. F. Fuortes (NINDB-O) regarding the interpretation of electric potentials recorded from eccentric cells of the Limulus eye. This consisted of comparing various hypothetical arrangements of membrane depolarization by low resistance shunts.

(3) Consulting with Drs. Karl Frank and M. G. F. Fuortes on the underlying theory and the interpretation of experiments involving the use of concentric intracellular micro-electrodes with electronic "voltage clamping" and other electrical boundary conditions. This is still in progress.

(4) Consulted with Dr. J. del Castillo (NINDB-EEG) on the interpretation of experiments with neuro-muscular junctions and experiments with single isolated nodes of Ranvier. This includes some calculations on quantal shunting of the nodal nerve membrane.

(5) Consulted with Dr. F. J. Brinley, Jr. (NINDB-LNP) on the distribution of current flow in cerebral cortex which results from a pair of point electrodes on the surface. A mathematical solution was provided. Also consulted by Brinley on several other mathematical questions.

Significance and Proposed Course: The Office of Mathematical Research, NIAMD, hopes to consult and collaborate with investigators in other Institutes and in a variety of subject areas. These examples illustrate that current neurophysiological research is an area in which such collaboration has been established. The trend of neurophysiological research is increasingly biophysical and the need for mathematical treatment is bound to increase.

Part I not included

Analysis of NIH Program Activities

January-December, 1958

National Institute of Arthritis and Metabolic Diseases.
Laboratory Research

INTRODUCTORY COMMENTS

In the office of the Associate Director in Charge of research, NIAMD, there hangs on the wall a framed quotation from Dr. Irving Langmuir, "Only a small part of scientific progress has resulted from a planned search for specific objectives. A much more important part has been made possible by the freedom of the scientist to follow his own curiosity in search of truth." In the pages which follow, the reader will find a number of examples of the kind of situation alluded to by Langmuir. Thus this year we can report that:

1. A physicist has discovered that a hitherto unrecognized by-product of its [illegible] reaction is of potent vasoconstrictor.

2. An organic chemist has made a notable contribution to the metabolic fate of an important hormone.

3. A mathematician has been able to supply a model for an enzyme-catalyzed reaction, pin-pointing the existence of a previously unsuspected [illegible].

4. A [illegible], studying [illegible] effects upon [illegible] drug metabolism by virus, has made a basic discovery [illegible] a possible new basis of therapy in [illegible] and [illegible].

These are but a few of the numerous contributions which might be cited to indicate the scope of free science and of free scientists within NIAMD. In each of these as well as in other cases, rigid disciplinary lines have been crossed to permit the scientist to exploit [illegible] the fullest his curiosity in some of these cases [illegible] Rather it would seem that the problem and the scientist grew and matured together, proceeding from one discussion to the next in logical sequence, regardless of [illegible] the techniques involved were those of mathematics, physics, chemistry, biology, or medicine.

This type of development is not to be confused with serendipity, which implies the accidental discovery of something actually being sought. Serendipity undoubtedly occurs and makes valuable contributions to the progress of scientific knowledge. In such advances, however, one can scarcely take much pride.

We do, on the other hand, view with pride those developments, such as the ones exemplified above, which have resulted from the venturesome adventures of our scientists beyond the confines of their immediate disciplinary backgrounds. This is the true exploration of science and may be distinguished as primary search, in contrast to the more humdrum research.

Scientific administration requires that there be some degree of planning. Undoubtedly the best planning is that which will provide, to the skillful investigator, a maximum of scientific freedom.

LABORATORY OF BIOCHEMISTRY AND METABOLISM

Carbohydrate Metabolism. Studies on the metabolism of [illegible] saccharides and oligosaccharides in liver have revealed the presence of a transglucosylase which does not [illegible] compounds. Glucosyl residues are transferred from maltose, maltotriose or glycogen to acceptors such as glucose or maltose, or to water. In the last case the reaction is irreversible.

The pathway of deoxyribose fermentation in Lactobacillus plantarum has now been delineated, and the enzymes involved have been partially purified. The first step, catalyzed by an adaptive deoxyribose kinase, is the formation of deoxyribose [illegible]-phosphate. The second step is reversible cleavage of this phosphate ester to acetaldehyde and triose phosphate by deoxyribose aldolase, which has now been purified about 100-fold. The enzyme was found to have a reasonable affinity for acetaldehyde. This fact removes the major objection to acetaldehyde as a precursor of deoxyribose phosphate. Isotope data suggest that [illegible] synthesis of deoxyribose in animal cells occurs by a C2-C3 condensation, which could be catalyzed by deoxyribose aldolase.

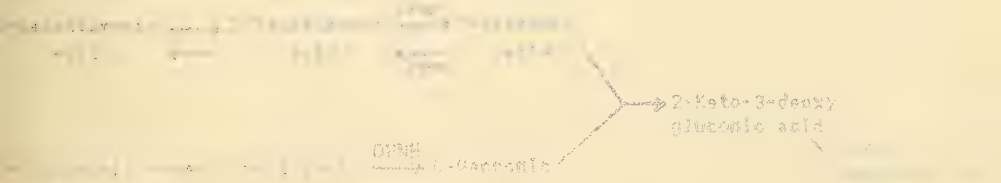
Studies on the physiological role of transketolase and [illegible] aldolase in reverse synthesis in animal tissues have [illegible] the presence of [illegible] ... [illegible] ... as for action, yet in liver supernatant fractions synthesis of ribose phosphate from hexose monophosphate occurs in the absence of added acceptors. This has also been found to be [illegible] ... [illegible] ... which must therefore act very efficiently in concert, each providing the triose phosphate or tetrose phosphate acceptor needed for the other

Work is being directed toward an elucidation of the chemical mechanism of galactose transport in E. coli. A mutant strain of this organism cannot metabolize galactose for lack of a kinase, but nevertheless concentrates this sugar within the cell. Apparently phosphorylation of the sugar is not essential to transport. The galactose transport mechanism is highly specific, requires energy and is poisoned by [illegible]. The free energy change for [illegible] has been calculated to be about 6000 calories per mole.

New evidence has been obtained for the existence of a [illegible] nucleotide which may serve as the precursor of this sugar in bacterial and blood-group polysaccharides. This nucleotide was isolated from a bacterial strain of Aerobacter [illegible]. It has been identified as [illegible] diphosphate [illegible] acetyl galactosamine. Aminosugars [illegible] are of [illegible] major importance for an understanding of the synthesis of complex polysaccharides, but also for the light it may shed on the origin of the deoxy sugars.

Further progress has been made in studies on the formation of uridine diphosphate galactose and its biological [illegible]. Evidence obtained with the purified UDP galactose epimerase from yeast indicates that this enzyme, like that from [illegible] liver, contains bound DPN present in a form with fluorescence resembling that of DPNH. The possible function of UDP galactose as a galactosyl donor for polysaccharides has been investigated; the preliminary results offer some promise.

A new reaction pathway for galacturonic and glucuronic [illegible] four intermediates which had not previously been known to be [illegible].



Studies on altered protein formation have revealed that in E. coli replacement of methionine by the analog norleucine does not prevent the formation of the adaptive enzyme β-galactosidase, even when 70 per cent of the normal methionine of the protein is replaced by norleucine. Other enzymes also appear at normal activity levels.

Nucleic Acid Metabolism. Polynucleotide phosphorylase which catalyzes polyribonucleotide synthesis in vitro has been further studied. The purified enzyme requires a primer to overcome a prolonged lag period in the polymerization reaction. Oligonucleotides with a 5'-phosphomonoester endgroup such as pApA, were found to be effective primers and these are incorporated into the beginning portion of the newly synthesized polynucleotide chain. It has now been shown that oligonucleotides of quite another kind are equally effective in overcoming the lag period and stimulating polymerization. These contain a 3'-phosphomonoester endgroup, and ApUp is an example. Such compounds cannot be incorporated into polymer and are recovered unchanged at the end of the reaction, but in their presence it is possible for polymerization to occur. This result is considered to be significant for it shows that formation of polyribonucleotides can be stimulated by a primer which need not be incorporated and therefore could act as a template.

A new activity has been discovered for polynucleotide phosphorylase, namely transnucleotidation, or the transfer of nucleotide residues from one polynucleotide to another.

It has been found that breakdown of polynucleotides by polynucleotide phosphorylase (phosphorolytic) or by snake venom diesterase (hydrolytic) proceeds in stepwise fashion, always from the same end of the molecule. This finding has provided a useful tool in studies, by others, on DNA synthesis and t-RNA function in protein synthesis.

Polyguanylic acid has been enzymatically synthesized for the first time. The mode of action of pancreas ribonuclease and liver nucleoside nucleotidase has been worked out.

Steroid Metabolism and Hormonal Effects. Steroid inactivation by saturation of the 4-5 double bond of ring A was found to be greatly increased in liver homogenates following treatment of the animals with thyroxin. This was attributed to the increased level of glucose-6-phosphate dehydrogenase

[illegible] made between the stimulating effect of steroids on glucose oxidation in the C-1 position with rat liver slices and the utilization of TPNH in the reduction of steroids. The hexose monophosphate shunt was stimulated only in tissues containing steroid reductases.

Efforts are being made to explain the stimulatory effect of certain steroid hormones on pyruvate oxidation in rat liver homogenates. This stimulation requires the addition of TPN which is absent in liver homogenates from fasted animals.

A search for a microbial 11-hydroxylation reaction has uncovered such a reaction in extracts of Rhizopus nigricans. It is hoped that the enzyme can be isolated and used for the study of the hydroxylation mechanism.

The mechanism of liver regulation of insulin level [illegible] insulin-I^{131} presented to liver is degraded in one passage through this organ. This suggests that the probable importance of liver in the maintenance of hormonal blood level and [illegible] the possibility of employing, for clinical purposes, active insulin derivatives which are not attacked by the insulin-inactivating system of liver.

Effect of Certain Steroids on the Metabolism of D-galactose [illegible] specific stimulatory effect on the conversion of D-galactose to CO_2 by liver and intestine slices. Recently a broken cell system has been obtained which manifests the hormone effect. In vivo manifestations of this phenomenon include the following observations: a) the onset of cataracts in rats maintained on a high galactose diet is delayed when the animals are treated with progesterone, and b) progesterone treatment endowed a galactosemic subject with some ability to convert D-galactose to CO_2.

[illegible] the existence of a condensation reaction between 2-methyl-4-amino-5-hydroxymethyl pyrimidine and 4-methyl-5-(β-hydroxyethyl) thiazole, catalyzed by extracts of baker's yeast. Two protein fractions are required, one to activate the pyrimidine and the second to catalyze the condensation.

[illegible] glucose to amino acids [illegible]. Simple model compounds, each containing the desired [illegible] groupings, were offered as sole carbon source, and by the enrichment culture technique, microorganisms were obtained with the ability to grow on these compounds. Enzymes were obtained from these organisms and reaction pathways worked out. Thus it was found that the initial step in the metabolism of acetylene mono- and dicarboxylic acids involves hydration with the formation of malonic semialdehyde and pyruvic acid, respectively. A vast number of acetylene derivatives are found in nature.

By similar techniques the enzymatic pathway for pyrrolidine was found to be: pyrrolidine ⟶ Δ^1-pyrroline ⟶ γ-aminobutyraldehyde ⟶ γ-aminobutyric acid ⟶ succinic semialdehyde ⟶ succinic acid. This investigation has led to the description of a system effective in the transamination and subsequent oxidation of γ-aminobutyric acid, an important reaction sequence of brain. It is also of interest that putrescine, formed in the gastrointestinal tract of mammals, can be metabolized by bacterial enzymes in a pattern similar to that used by pyrrolidine; namely, putrescine ⟶ γ-aminosemialdehyde ⟶ γ-aminobutyric acid ⟶ succinic semialdehyde ⟶ succinic acid.

Cyclic alcohols have been employed as models for steroid metabolism, with the finding that cyclohexanol and its methyl derivatives are excellent substrates for horse liver alcohol dehydrogenase. These studies may shed more light on the physiological function of this powerful enzyme and also on its steric specificity.

Enzyme Mechanisms. Experiments with H_2O^{18} have established [illegible] cleavage of the glucuronides [illegible] of β-glucuronidase at the bond between the anomeric carbon atom of glucuronic acid and oxygen, since only the glucuronic acid becomes labeled. The same pattern is observed with acetal or ester type glucuronides. Concurrent studies with labeled inositol indicate that this substance may indeed serve as a precursor of glucuronic acid.

Further work in progress is designed to elucidate the mode of action of aldolase and triose phosphate isomerase.

Studies on Tumor Tissues. [illegible] of phosphorylase have been studied in various tumor tissues. The enzyme is absent in the Novikoff hepatoma, whereas certain muscle sarcomas contain [illegible] levels. It is also absent in ascites tumors which do, however

[illegible] ...ed ... [illegible] ...
... [illegible] ... Jack in the normal hormonally-induced sequence of [illegible] ... activation. The finding of a difference in [illegible] ... in [illegible] pathway helps to clarify the unknown factors which control the extraordinarily high rate of carbohydrate utilization of tumor cells.

Preliminary results also indicate a steroid inhibition of hexose utilization either prior to the formation of 6-phosphate or on both the glycolytic and oxidative pathways of glucose degradation. It will be of interest to extend this observation to the "steroid-in-sensitive" tumors, to explain whether the resistance to steroids represents a survival of previously existing resistant cells or a steroid induced change in the genetic character of the sensitive cells.

LABORATORY OF PHARMACOLOGY & TOXICOLOGY

Shock and Infections. Gamma globulin of various species of animals was shown to be highly effective in preventing [illegible] from organisms of low virulence (Pseudomonas, Coli and Staphylococcus). While believed to be due to "natural antibodies," high protective titres were also demonstrated in the sera of germ-free rats. Greatly increased potency was obtained in sera from animals immunized with pseudomonas and staphylococci. [illegible] rabbit immune globulin is being prepared for clinical trials against pseudomonas infections. A general correlation between hemaglutinin titre and protective action was established.

The Peru Project continues to show the efficacy of oral sodium solutions in burn shock in adults (80 cases with no deaths from shock) while in 70 children saline + plasma was slightly superior to saline alone, in a similar series. After one year's trial the prophylactic use of gamma globulin [illegible] systemic pseudomonas and staphylococcus infections in human burns indicates a favorable effect, which must be confirmed in a larger series of cases.

Using the quantitative urinary excretion of steroids following ACTH infusions as a measure of adrenal cortex activity, it was shown that severe burns produce maximum stimulation, since no further increase in excretion is brought about by ACTH.

Assay of several drugs in mouse leprosy showed [illegible] to have promising activity. Growth of M. lepraemurium has been achieved in tissue culture of monocytes.

for human and animal consumption.

shown that Ca entered stimulated muscle fibers proportional to the stimulation, while no increased entry occurred in resting muscle. The implication of Ca in muscle contraction is suggested. By studying the fluxes of Ca in resting muscle and tendon under various conditions, a nonspecific binding site for Ca was shown in muscle that is not present in tendon.

Basic Proteins. Quantitative studies of the basic proteins in tissues (protamine and histones) have been stimulated by development of chromatographic techniques for their isolation. The histones may be eluted from ion exchange resins by sodium lauryl sulfate, while the protamines can be eluted from certain resins by acids. Rapid methods have been devised which are applicable to whole tissue extracts.

Amine Metabolism. The steps in the biosynthesis of spermidine from putrescine and methionine have been further elucidated. The three enzymes from E. coli have been partially purified, and the reactions characterized. All but one of the end products have been isolated.

Putrescine and spermidine have been identified as basic constituents of bacteriophage T4, in amounts sufficient to neutralize about half of the phosphate groups of the nucleic acid. The relation of these amines to genetic function is being investigated.

In E. coli grown at alkaline pH, acetylation of these amines has been demonstrated. Acetylation is stimulated by addition of the amines to the medium, and acetyl derivatives of putrescine, spermidine and spermine have been identified chromatographically. This observation is of particular interest since this type of acetylation is unusual in bacteria.

Amino Acid Metabolism. Histidine. The degradation of histidine by enzymes purified from liver has been further elucidated. The pathway proceeds through urocanic acid to formimino glutamic acid (Figlu). The pathway of Figlu is as follows:

1) Figlu + tetrahydrofolic acid (TFH) $\rightleftharpoons$ formimino TFH + glutamic
2) Formimino TFH $\rightarrow$ 5,10-methenyl TFH + NH_3
3) 5,10-Methenyl TFH $\rightleftharpoons$ 10-Formyl TFH

[illegible] methods for determination of [illegible] have been described, and a report has been published showing marked increases of [illegible] excretion in cancer cases after treatment with [illegible] acid drugs.

Histamine. Imidazolacetic acid riboside has been demonstrated in the urine after histamine or imidazolacetic acid administration. This compound has now been synthesized, and shown to be identical with the natural product.

Tryptophan. The previously reported increase of [illegible] liver of diabetics of the enzyme [illegible] concerned in the degradation of tryptophan, has been further [illegible]. The increase of this enzyme is not primarily related to [illegible] carbohydrate utilization, but is influenced by several [illegible]. Increase may be obtained with cortisone administration [illegible] effect is opposed by insulin + growth hormone, but [illegible] either alone. The cortisone effect is also opposed by [illegible].

Sulfur Amino Acids. A study of sulfur metabolism [illegible] has revealed several new sulfur containing compounds, and [illegible] metabolic pathways of sulfur amino acids have been elucidated. [illegible] enzyme systems have been isolated which reduce the [illegible] methionine sulfoxide. One system is specific for the [illegible] isomers, another for the L- and D- isomers. TPNH is [illegible]. The sulfur amino acid felinine appears in large amounts [illegible] urine of feline species. By S^{35} and C^{14} labels, it has been shown that cysteine and mevalonic acid are precursors.

[illegible] polypeptide of 6 amino acids, is being studied [illegible] free extract of B. brevis. Using C^{14} label, a compound has been formed with an amino acid composition similar to [illegible] biotic, but which is not identical. The various [illegible] been established. Since little is known of polypeptide synthesis, attempts are being made to establish whether [illegible] ds through a stepwise linkage or through a "template" [illegible] view.

Sialic Acid. A more sensitive and more specific method [illegible] estimation of sialic acid has been devised. High concentrations of sialic acid have been found in human semen, seminal

Antibody localization. [illegible] of reagglutinable [illegible] with fluorescein tagged [illegible] technique, the distribution of hyaluronidase in mouse tissues and of myosin in muscle fibers has been demonstrated.

LABORATORY OF PATHOLOGY AND HISTOCHEMISTRY

Hematologic Studies. Investigation of the factors [illegible]

Studies in this laboratory have quantified certain of the [illegible] involved. The severity of hypoxia governs the rate of production of erythropoietine. The rate of utilization is influenced by the bone marrow cellularity. The balance of these two factors determines the plasma level. Erythropoietine appears in the urine if the plasma level is sufficiently high; plasma and urine erythropoietine appear chemically identical. A [illegible] mechanism for the control of erythropoiesis has been postulated: the hypoxia-erythropoietine complex and a second mechanism yet to be defined. The existence of a second mechanism of control was postulated as the result of clinical and experimental observations of alterations in the rate of red cell production in the absence of absolute or relative changes in O_2 delivery or demonstrable erythropoietine production. The working hypothesis that has been advanced for the second mechanism is that an [illegible] regulator of erythropoiesis is produced by senescent red cells released on the death of the cell. Increase in red cell production is achieved through increase in the number of new [illegible] progenitor cells together with a decrease in the normal proportion of marrow normoblasts, rather than by a reduction in the [illegible] mitotic interval of maturation time.

In vitro labeling of marrow and peripheral blood with [illegible] tritiated thymidine demonstrated the expected high proportion of cells in the stage of DNA synthesis in normal marrow; the presence of occasional mononuclear cells in DNA synthesis in the peripheral blood of normals; an increased number (up to 5%) of such cells in certain infections, particularly of childhood; and a surprisingly low proportion (often less than 5%) of [illegible] labeled "blast" cells in acute leukemias.

Studies of the physical and chemical properties of hemoglobin have shown that each molecule of hemoglobin dissociates

as well. Recombined, each polypeptide chain of the hemoglobin differs, a double exchange of fragments can be made, as was done with a mixture of human and canine hemoglobin. These results show one of the ways in which a molecule is built up of its constituent parts and also contribute to our knowledge concerning the [illegible] protein structure and the relationship of inherited alterations in protein structure to disease. Further attempts will be made to produce pairs of unlike peptides, and to determine structural relationships of hybrid molecules to the intermediate compounds of hemoglobin reactions. Study of sulfhydryl groups of hemoglobin is underway to determine the extent to which they may explain the specific chemical difference between normal adult hemoglobin and an abnormal hemoglobin I will be investigated.

Experimental Arthritis. Studies of the factors in degenerative joint disease in laboratory animals is being continued, particularly with respect to the role of [illegible] hybrids, back crosses and reciprocals of certain mice. It is evident that osteoarthritis of mice is genetically influenced and behaves as a recessive. From expanded work it is expected that the number of genes involved can be determined. In studying potentiating factors for osteoarthritis of mice, whole body irradiation was found to have no such effect. The production of arthritis of mice that resembles the vascular lesions of human rheumatoid disease is under study, a large colony of susceptible mice [illegible] is being developed.

Experimental osteoarthritis in rats produced by infection with Streptobacillus moniliformis has been defined in detailed pathological and bacteriological terms. Two serological reactions for human rheumatoid arthritis, the sheep cell agglutination test and the bentonite flocculation test, have been positive in these infected animals. These tests have also been positive in rats immunized with S. moniliformis antigens, indicating that they are an immunologic response, independent of joint inflammation or infection. S. moniliformis has been isolated from the blood of a patient with high fever and no history of rat bite. This patient's serum has shown an elevated sheep cell agglutination titre. An atypical PPLO has been isolated from the blood, joint, and conjunctiva of a patient with [illegible] transforming factor of rabbit myxoma virus for the rabbit

infectious processes. A survey of all available ... brain which rendered rabbit ... of allergic encephalitis in guinea pigs. Since encephalitis ... occurs occasionally in man following use of such vaccines ... similar studies using infected brain of young rabbits (not allergic) are being investigated; preliminary results ... encouraging. In connection with polio vaccine safety ... significant lesions have been found in 10 of 1560 monkeys ... three, pathologic lesions were characteristic of specific ... pathologic agents and confirmed by tissue culture. In view of ... growing awareness in live virus polio vaccines, the ... pathology produced by various attenuated strains is being ... studied and standard methods of grading the degree of attenuation are being developed. Attempt to infect paralysis ... polio virus was unsuccessful. A human case was reported ... to such a source.

Pulmonary adenomatoid-like lesions were induced in ... suckling squirrels by squirrel fibroma virus. A virus ... for similar lesions in man and certain other animals has ... suspected but never proven. Investigations were carried ... the nature of lesions induced by a new "monkey tumor virus." ... nodules of suckling rabbits that follow subcutaneous ... inoculation with the Sivemart polyoma virus, and the pathology ... case of mice that follows infection with the Friend ... agent. In the latter, the spleen is first involved but the ... process spreads extensively to involve the liver, bone ... marrow and occasionally other organs, and histologically ... indistinguishable from a malignant process.

Stress and Endocrine Effects. Dogs given a subcutaneous ... injection of epinephrine-in-oil (1 mg/kg) show a sharp rise ...

and sedimentation constants of myosin prepared
al and failing hearts of dogs. Studies on the
ed by fiber bundles of glycerol-treated muscle
not directly correlated with the energy the
liable from the splitting of the terminal

A method has been adapted for the analysis of
in blood sera in the presence of calcium
and dye chrome fast blue BF.

Preliminary data indicate that part of the phosphate
myosin is bound to the amino acid serine. Further
this localization is under way. By a technique
activation of thrombin it was shown that radioactively
phosphate was bound to serine located in the sequence
ser-PO_4-gly. This investigation establishes
sequences in the active site in a number of
and clearly implies that a specific serine residue
in the hydrolytic activity of these enzymes

cyanidine, the acyl shift in proteins has been analyzed
new mechanism for the aggregation of proteins and a new
for general proteolytic enzyme action has also
the acyl shift. An understanding of the
action

Preliminary findings indicate that all
changes are clustered in the N-terminal vicinity
It is postulated that this may specifically
to a definite point of hydrolysis or the fibrinogen
by imparting high local negativity to the
be the means by which fibrinogen molecules are
in the unclotted state. Model compounds such

The net scientific production of NIAMD continues [illegible] abundant in quantity and of high quality. Collaborative [illegible] which cross laboratory and institute lines continue [illegible] develop and prosper. New lines of investigation are [illegible] being developed, new investigators are being recruited, [illegible] far as space permits. Plans for progressive future [illegible] growth are being given active consideration.

The intramural educational program is also a matter [illegible] gratifying interest. The Research Associate program, [illegible] was initiated in July, 1957, now has three incumbents [illegible] this number will go up to six in July, 1959, and to [illegible] 1960. Candidates of exceptionally high caliber [illegible] continue to apply for these positions and the program is [illegible] an important part of the Institute's activities. [illegible] six fellows in the COSTEP program and five in [illegible] High School Science Teachers' program have been [illegible] positive [illegible] to disseminate the knowledge and research skills accumulated [illegible] to an appreciative and fertile audience. They also [illegible] [illegible]

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

ANNUAL PROJECT REPORT

CALENDAR YEAR 1958

Summary Sheet

ARTHRITIS AND RHEUMATISM BRANCH

Estimated Obligations for FY 1959

Total - $555,414
Direct - $338,000
Reimbursement - $217,414

Project Numbers 117-C thru 127-C Included

Serial No. NIAMD - ARC-C
1. Clinical Investigations
2. Arthritis and Rheumatism
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: The Study of Reiter's Syndrome

Principal Investigator: Dr. John R. David

Other Investigators: Dr. Joseph J. Bunim

Cooperating Units: Drs. Edwin Lerner II and Leon Sokoloff (LPH), 02
Elizabeth Jackson (NIAID),
Fletcher P. Williams, Jr. (DRS),
Drs. Patricia Kramer and Ruth Wittler(Walter
Reed Institute of Research) and
Dr. Eldon G. Kronewitter (Preventive and Chronic
Disease Division, Dist. of Col., DHEW)

Man Years (calendar year 1958):
Total: 2/3
Professional: 2/3
Other: 0

Project Description:

Objectives:

1) A clinical study of the syndrome in order to learn more about the diagnostic features and the natural course of the disease.

2) To attempt to isolate the etiological infective agent.

3) To define the joint pathology.

Methods Employed and Patient Material: Patients with Reiter's syndrome were completely worked up as to history, physical examination, laboratory data and x-rays. Samples of blood, synovial fluid, conjunctival, and urethral exudate were studied for microorganisms. Specially enriched media were used as well as tissue culture. Synovial biopsy specimens were studied for micro-organisms and given to pathology.

Part B included Yes [ ] No [X]

Major Findings: We have not been able to isolate any micro-organisms that can be related causally to the syndrome. Further use of tissue culture, however, may prove rewarding. Biopsies of the synovial membrane showed a non-specific inflammatory process.

Significance to NIAMD Research: The importance of Reiter's syndrome as an increasingly common type of arthritis and its frequent confusion with rheumatoid arthritis make it important to NIAMD research.

Serial No. NIAMD - 110-C
1. Clinical Investigations
2. Arthritis and Rheumatism
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: The Bentonite Flocculation Test in Rheumatoid Arthritis

Principal Investigator: Dr. Kurt J. Bloch

Other Investigators: Dr. Joseph J. Bunim

Cooperating Units: Dr. Robert R. Williams (LPB) 10%
Dr. Edwin Lerner II (LPH) 7%

Man Years (calendar year 1958): Patient Days (calendar year 1958):
Total: 1-1/2
Professional: 5/6
Other: 2/3

Project Description:

Objectives: To continue the evaluation of the Bentonite Flocculation Test (BFT) as a diagnostic aid in rheumatoid arthritis.

Methods Employed; Major Findings:

Sensitivity and Specificity of the Bentonite Flocculation Test:

Evaluation of the sensitivity and specificity of the BFT as a serologic tool in rheumatoid arthritis has been continued in the past year. A total of 152 patients with definite rheumatoid arthritis were tested; of these 130 or 86% were positive. Among the last 71 patients with definite rheumatoid arthritis examined by NIH staff members, 90% gave positive tests. Five patients with probable rheumatoid arthritis had negative tests. Fifteen patients with juvenile rheumatoid arthritis were tested; of these only one had a positive BFT. This patient had onset of arthritis in childhood with persistent disease at age 42. The collagen diseases contributed the next highest incidence of positive tests. In systemic lupus erythematosus 9 of 15 patients tested were positive; 5 of 10 patients with scleroderma were positive; 4 patients with dermatomyositis had negative tests.

Part B included Yes [X] No [ ]

Patients with other types of arthritis were also tested. Eleven patients with rheumatic fever had negative tanned-cell flocculation tests, 34 gout patients were tested; only one had a positive test. Nine patients with osteoarthritis had negative tests. One of 13 patients with Reiter's syndrome had a positive test. Three patients with rheumatoid spondylitis had negative tests. The test was negative in four patients with psoriatic arthritis.

The RFT in Diseases in which Macroglobulins Occur:

The rheumatoid factor is a high molecular weight gamma globulin. Some Wassermann antibodies, cold agglutinins, complete Rh antibodies, iso-agglutinins and the macroglobulins of Waldenstrom's macroglobulinemia belong to the group of proteins. About 10% of syphilitic patients as well as patients with biological false positive reactions had positive test results. Two of 5 patients with macroglobulinemia of Waldenstrom were positive.

The RFT in Normal Volunteers, Blood Donors and Relatives of Patients with Rheumatoid Arthritis:

Sixty-five normal volunteers were tested; of these one had a positive test. There were 9 positive tests among 365 blood donors. Generally, it is reported that 2 - 3% of normal individuals have positive serologic tests for rheumatoid arthritis. Seventy-six relatives of patients with rheumatoid arthritis were tested; of these had a positive test.

Use of the RFT in Epidemiological Screening:

In cooperation with a USPHS Health Survey Pilot Study, sera from 112 individuals were tested; two doubtful and one definite positive result were obtained. We examined and tested a group of 20 New York families enrolled in a Family Health Demonstration Unit; no positive RFT results were obtained, although 2 cases of possible rheumatoid arthritis were found. Dr. Baruch S. Blumberg examined 109 Wainright Eskimos. A surprisingly high incidence of positive tests were encountered - 11.8%.

The RFT in Body Fluids Other Than Serum:

Synovial fluid from 5 rheumatoid patients was tested. In these five patients the RFT was positive, and the titer tended to be lower than that obtained with the patients' serum. Cerebrospinal fluid from 3 patients with rheumatoid arthritis who had moderate and high serum RFT titers yielded negative tests.

Long-term Serial BFT Determinations on Rheumatoid Patients:

The BFT has been performed, at weekly intervals, on all rheumatoid patients admitted to our Unit. The serological results will be correlated with disease activity, treatment and prognosis.

Whole Serum Inhibition of the Reaction between Gamma Globulin Coated Bentonite and Rheumatoid Serum:

Grubb, in Sweden, demonstrated that 60% of sera from normal donors inhibited the reaction between rheumatoid serum and human group O, Rh positive red blood cells coated with incomplete Rh antibodies. Inhibition or failure of inhibition was found by Grubb to be a genetically determined property of serum. A whole serum inhibition procedure employing coated bentonite, in place of coated red blood cells was developed in our laboratory. It was found that about 60% of normal sera inhibited the flocculation of bentonite particles by a diluted BFT positive rheumatoid serum. Forty per cent of normal sera failed to inhibit the flocculation reaction. Tests performed on the sera from 28 New York families tend to confirm the genetic hypothesis.

It was anticipated that the substitution of coated bentonite particles for incomplete antibody coated human erythrocytes would provide a simpler test system. Experience indicates, however, that the present whole serum inhibition procedure is not entirely satisfactory because of difficulty in finding appropriate gamma globulin preparations and appropriate rheumatoid sera to be inhibited.

The BFT in Experimental Arthritis:

In cooperation with Dr. E. Lerner of this Institute, the BFT was performed on serum from rats experimentally infected with Streptobacillus moniliformis. This organism produces bone and joint lesions. It was found that BFT titers in infected animals were significantly higher than those in control animals.

Isolation of the Rheumatoid Factor:

In cooperation with Dr. Robert R. Williams, Jr. of this Institute, attempts were made to isolate the rheumatoid factor employing three different procedures. In the first procedure, a complex was formed between serum rheumatoid factor and heated normal human gamma globulin. The complex was recovered and dissociated with an acid buffer. The resulting solution was placed on a sucrose gradient and subjected to ultracentrifugation. The high molecular weight rheumatoid factor was recovered from the bottom fraction. This procedure resulted in a preparation with several hundred-fold increase in specific activity (measured by the Newcastle Disease Virus inhibition procedure) compared to the starting serum.

A second approach employed Cohn method 10 for fractionating serum. The fraction containing the rheumatoid factor was then placed on a carboxymethylcellulose (C.M.C.) column. Subsequent elution with buffer solutions produced a fairly pure preparation of rheumatoid factor. However, contaminating serum proteins were still present.

A third approach involved a one-step method of serum fractionation developed by Dr. H. Saroff of the Section on Physical Biochemistry. Material obtained by this procedure was further fractionated on a C.M.C. column. Again considerable purification of the rheumatoid factor was achieved, but contaminating proteins were not entirely removed.

Significance to NIAMD Research: The BFT is a valuable laboratory aid in the diagnosis of rheumatoid arthritis. It is a simple, rapid, and reliable procedure and for these reasons easily adapted to epidemiological and other studies.

Proposed Course of Project:

a). Several authors have reported that healthy family members of patients with rheumatoid arthritis have a higher incidence of positive tests for rheumatoid arthritis than normal control families. Since the BFT fails to detect these family members, other test procedures, currently available, will be tried.

b). We plan to study the effect on the BFT of various immunological procedures, including hyperimmunisation, in animals and man.

c). We propose to continue the study of the relationship between high BFT titer and "complications" of rheumatoid arthritis. These "complications" include splenomegaly, anemia, leukopenia, vasculitis, neuropathy and Sjogren's syndrome.

d). Experiments with dyes, other than methylene blue, for staining the coated bentonite particles may result in improvement of the test procedure.

e). A combination of the one-step chemical serum fractionation method followed by density-gradient ultracentrifugation may result in further purification of the rheumatoid factor.

Arthritis
Individual Project Report
Calendar Year 1958

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

1) Bloch, K. J., and Gunsin, J. J.: Simple, rapid diagnostic test for rheumatoid arthritis -- bentonite flocculation test. (in press).

Honors and awards relating to this project:

There were no honors or awards.

1. Clinical Investigations
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Genetic Polymorphisms in Man and Other Animals

Principal Investigator: Dr. Baruch S. Blumberg

Other Investigators: Dr. Kurt J. Bloch, Dr. Jacob Robbins and Dr. Joseph J. Bunim

Cooperating Units: Dr. A. C. Allison, National Institute for Medical Research, London; Dr. Stanley Gartler, University of Washington, Seattle; and Dr. F. H. Allen, Blood Grouping Lab., Boston; Dr. R. Kellermeyer, Joliet, Illinois, and Dr. T. von Brand, NIAID

Man Years (calendar year 1958)
Total: 2
Professional: 1
Other: 1

Projection Description:

Objectives: To determine the presence of biochemical polymorphisms in man and other animals and to investigate their distribution in different populations. To apply the findings to problems in arthritis.

Methods Employed; Major Findings:

General - Many biochemical traits in humans and other animals are genetically determined. Some of these may be classified as genetic polymorphisms; that is, the existence in a population of two or more easily distinguished forms of a trait, the lesser of which could not be maintained by recurrent mutations alone. The normal hemoglobin-sickle cell hemoglobin system, most of the blood groups, and other systems to be described below, fall within this classification. From studies in lower animals and theoretical considerations, there is reason to believe that selection may operate to maintain these traits in the incidences found and in some cases the selective forces may be related to disease. It is of interest to determine if populations living under different environmental conditions and prone to different diseases have different incidences of these genes. In addition studies of the distribution of these traits can sometimes provide information on genetic relations between separated population groups. A field trip to Northern Alaska was made in the summer of 1958.

Part B included Yes [X] No [ ]

1) Haptoglobins

These are a family of serum proteins which bind hemoglobin. They occur in three major patterns in humans and their presence or absence is genetically determined. Blood collections from Eskimos and Athabascan Indians were made during the Alaska field trip. Bloods from Micronesians were supplied through the courtesy of Dr. J. E. Hall. It was found that Eskimos and Indians living under similar environmental conditions have distinctly different distributions of the phenotypes. The Eskimos have a much lower incidence of the type 1-1 than do European populations (Eskimos - 7%, Europeans - 15%) and a very much lower incidence than that found in West Africans during our field trip of 1957 (West Africans - 50%). Micronesians from the Marshall Islands have an incidence of type 1-1 of 41%, and Alaskan Indians approximately the same as Europeans. These differences may be explained in terms of "racial" dissimilarity as well as possible differences in selections for this trait. Several haptoglobin types, not previously described in man, were discovered in the Eskimo, Indian, and Micronesian populations. These may represent rare genetic re-combinations.

The sera of several animal species have been investigated to determine if they possess haptoglobins and if so, if they form a polymorphic system. Bloods from approximately 100 Pribilof fur seals, Arctic ground squirrels and marmots were collected during the Alaska field trip. All these were found to have haptoglobins and intraspecies differences were found in the seal and marmot. By analogy to the findings in humans it is likely that these haptoglobin types are also genetically determined. An investigation of the sera of laboratory rats has been initiated. They appear to have more than one haptoglobin type. If this is confirmed in subsequent studies and a genetic determination of the types demonstrated, they may provide suitable subjects for the study of selection in the haptoglobin system.

2) Gamma Globulin Groups

The agglutination test for "rheumatoid factor" has been used as a diagnostic method for rheumatoid arthritis. It has recently been shown by Grubb that the sera of some humans will inhibit this reaction, and that the inhibiting material travels in the gamma globulin fraction. This inhibiting property is genetically determined by a single autosomal gene. To date these studies have not been confirmed in America. In collaboration with Drs. Kurt J. Bloch and Joseph J. Bunim, we have investigated this system, using the Bentonite Flocculation Test. Family studies were conducted in New York by Dr. Bloch, and blood group, haptoglobin, taste tests and gamma globulin determinations were performed on blood collected from the family members. The preliminary results appear to confirm the findings of Grubb. We hope to conclude this work and standardize the test for routine use on sera from other populations (i.e. Eskimos and Indians).

3) Urinary β-Aminoisobutyric Acid (BAIB) Excretion

It has been shown by Gartler and others that the excretion of BAIB is in part under genetic control. Some persons with leukemia and other cancers are also high excretors, but the genetic role in these cases is not clear. Individuals who excrete large amounts of BAIB are rare in European populations. Approximately 150 urine samples from Alaskan Eskimos and Indians were studied with the collaboration of Dr. A. C. Allison and Dr. Stanley Gartler. It was found that more than half of the Indians and one-fourth of the Eskimos are high excretors compared to only 10% high excretors in Europeans. The reasons for these differences are not known.

4) Taste Test

The ability to taste phenylthiocarbimide (PTC) and related compounds in certain concentrations is genetically determined, although the threshold distinguishing tasters from non-tasters may vary in different populations. PTC and related substances are chemically similar to some goiterogenic materials and it has been suggested that there is an association between the taste-non-taste polymorphisms and thyroid disease. By means of a selection test using 7 serial dilutions of PTC the incidence of non-tasters in Eskimos was found to be 26%. This is the first time that a properly controlled study has been done on this population. The gene frequency is distinctly different from that of North American Indians and is another indication of the genetic disparity between the Eskimo and Indian Groups. This work was done in collaboration with Dr. A. C. Allison.

5) The Primaquine Sensitive Red Blood Cell Test

Ingestion of primaquine and related compounds leads to a self-limited hemolytic crisis in about 10% of American Negroes. It has been found that this is due to a deficiency in glucose-6-phosphate dehydrogenase and other enzymes, and that the deficiency is under genetic control. This trait is also found in Mediterranean Jews (and makes them susceptible to Favism) but is very rare in other Europeans. Studies in other population groups have not yet been reported. In collaboration with Drs. R. Kellermyer and A. C. Allison the presence of this trait in Alaskan aborigines was investigated. Two of 60 Eskimos studied were found to have the deficiency. We are now attempting to confirm this finding. It is hoped that with further information on the distribution of this trait it will be possible to find some clues as to the selective forces which maintain this enzymatic deficiency in the population.

6) Blood Group Studies

In conjunction with the other genetic traits the incidence of blood groups in Alaskan populations has been studied (in collaboration with Drs. F. H. Allen and A. C. Allison). Thirty-seven separate antigens were used. The findings, in general, confirm those of previous investigators. The incidence of the Diego factor was studied

in these groups for the first time. This blood group is absent in Europeans and Africans but common in South American Indians and in lesser incidence, in North American Indians. It is also present in Japanese and Chinese populations and in some Southeast Asian groups. We found a very low incidence of this trait in Athabascan Indians (less than 1%). This is the lowest incidence so far found in any American Indian population. If these findings are confirmed in subsequent studies in North and South American Indian populations, it would suggest that a North-South cline exists for this trait. It is possible that selection may have operated to cause this distribution.

7) Studies with the Starch Gel Electrophoresis Method

We have initiated studies of the localization of the rheumatoid factor by means of starch gel electrophoresis. This method yields a separation of serum proteins dependent, not only on charge but also on molecular size. Our early runs have indicated that the rheumatoid factor molecule or molecules are very large or perhaps elongated and that they enter the gel only with difficulty. It is not associated with of the genetically determined proteins described to date We hope to be able to develop a preparative technique for separating larger amounts of rheumatoid factor than possible using the methods currently available

In collaboration with Dr. Jacob Robbins we have studied the localization of radioactive thyroxin in serum. There are three bands in the albumin and one in the pre-albumin which bind thyroxin. There are differences in thyroxin bindings in some individuals and further studies will be required to determine the significance of this. A new method of radio autography of starch gel, hitherto not used, has been developed to deal with this problem.

8) Genetic and Other Studies in Arthritis

The studies on rheumatoid factor and the gamma globulin groups have been described above. We are currently collecting data for the study of the incidence of arthritis and the rheumatoid factor in families. A survey of the incidence of arthritis and rheumatoid factor in a small Eskimo village has been completed.

9) General Studies in Human Genetics

In collaboration with Dr. A. C. Allison two studies on general human genetics have been completed. These include a critique on dominance and recessivity in man and a comprehensive survey of genetic polymorphisms in man.

10) Animal Hemoglobins

The hemoglobins of some animals have been studied as an example of a polymorphic system. It has been shown that some cattle breeds contained individuals which have two hemoglobin types (A & B). During a 1947 West African field trip we found that the dwarf

"coast cattle" did not have this polymorphism while the humped, Zebu breeds of the interior do. These studies were completed this year. We have surveyed and typed a large number of the dairy herd at the Agricultural Research Station, Beltsville, Maryland, through the courtesy of Dr. J. F. Sykes. Our African studies suggested that there might be some relation between the polymorphism and resistance to hypanosomiasis. With the cooperation of Dr. Theodore von Brand the effect of the different cattle hemoglobins on the cultural properties of African hypanosomes is being studied. We are continuing our typing of local cattle to find animals for this study.

The hemoglobin systems of the seal, marmot and ground squirrel have been studied. The seals have two major and one minor peak on electrophoresis and two peaks can sometimes be resolved in the marmot hemoglobins. No intraspecies variations were found in any of these animal groups.

Proposed Course of Project:

1. Completion of family studies on the incidence of rheumatoid arthritis and its possible correlation with other genetic traits.

2. A study of the genetically determined beta globulins in humans.

3. A study of the genetically determined cholinesterases of serum.

4. A field trip to the Central Pacific for the investigation of a Micronesian population which has been subjected to high radiation levels due to fallout. A study of the incidence of certain genetic traits and of rheumatoid arthritis will also be attempted in this group.

5. A study of the incidence of genetic biochemical traits in experimental animals to facilitate breeding and selection experiments

PHS-NIH
Individual Project Report
Calendar Year 1958

Part B:

Honors, awards and publications

Publications other than abstracts from this project:

1) Allison, A. C., and Blumberg B. S.: Genetically determined serum haptoglobins in rheumatoid arthritis. Arth. & Rheum. 1: 239-243, 1958.

2) Bangham, A. D., and Blumberg, B. S.: Distribution of electrophoretically determined hemoglobins among some cattle breeds of Europe and Africa. Nature, 181:1551-1552, May 31, 1958.

3) Blumberg, B. S.: Joint lubrication. Bull. on Rheum. Dis. 9: 169-170, October, 1958.

4) Blumberg, B. S.: Book review - "Natural selection in man". Eugenics Quarterly. 5: 171-172, 1958.

5) Corcoran, B., Allen, F. H., Allison, A. C., and Blumberg, B. S.: Blood groups of Alaskan Eskimos and Indians (in press).

6) Gartler, S. G., Blumberg, B. S., and Allison, A. C.: Urinary β-aminoisobutyric acid excretion in Alaskan Eskimo and Indian populations. Nature (in press).

7) Blumberg, B. S.: Bernard Connor's description of the pathology of ankylosing spondylitis. Arth. & Rheum. 1: 553-563, 1958.

8) Allison, A. C., and Blumberg, B. S.: Dominance and recessivity in medical genetics. Am. J. Med. 25: 933-941, 1958.

Serial No. NIAMD - 129-C
1. Clinical Investigations
2. Arthritis and Rheumatism
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Study of Familial Generalized Osteoarthritis

Principal Investigator: Dr. Roger L. Black

Other Investigators: There were no other investigators in the National Institute of Arthritis and Metabolic Diseases

Cooperating Units: Dr. Carl Witkop (NIDR)

Man Years (calendar year 1958):
Total: 2/3
Professional: 1/3
Other: 1/3

Project Description:

Objectives: Since very little is known about the cause, predisposing factors, or even the course of osteoarthritis, a detailed study of a large family having a high prevalence of osteoarthritis was begun in order to learn something of the genetic factors involved.

Methods Employed and Patient Material: A family of approximately 400 individuals is being evaluated for the occurrence of generalized osteoarthritis. This family, living in a limited geographic area, has a history of some degree of in-breeding. These people have had an unusual incidence of "back trouble" and tend to be short and obese.

This study has been designed strictly on an outpatient basis and involves careful history taking, physical examinations, and x-ray, and clinical laboratory studies. Genetic data is being collected by Dr. Witkop.

Part B included Yes [ ] No [X]

Major Findings: Forty individuals in this family have been studied in 1958. The findings in these 40 are as follows:

	Age	M	F	Total
Group A	Under 20	1		1
Group B	20 - 39	2	15	17
Group C	40 - 75	6	16	22
				40

In Group A the one patient showed no disease.

In Group B osteoarthritis of the spine was noted in one of the 2 males, and in 6 of the 15 females.

In Group C 5 of the 6 males had spine changes, as did 14 of the 16 females.

The observations reported show, in general, similarity to those in the first 52 patients reported last year.

This group is also serving as a control group for x-ray observations of the cervical spine in rheumatoid arthritis patients.

Significance to NIAMD Research and Proposed Course of Project:

The importance of osteoarthritis as a common disease and as a cause of loss of time in industry makes it important that the NIAMD continue an interest in some aspects of this disease.

It is expected that more of this large family will be evaluated in the forthcoming year and that correlations between obesity, short stature, and this disease can be made in this family.

Serial No. NIAMD - 121-C
1. Clinical Investigations
2. Arthritis and Rheumatism
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Effects of Steroids on Carbohydrate Metabolism

Principal Investigator: Dr. K. Lemone Yielding

Other Investigators: Dr. Gordon M. Tomkins

Cooperating Units: There were no other cooperating units in the Public Health Service

Man Years (calendar year 1958):
Total: 5/6
Professional: 1/2
Other: 1/3

Project Description:

Objectives: To observe effects of various steroids on in vitro metabolism of carbohydrate, ultimately attempting to define mechanisms of action at the molecular level.

Methods Employed: Tissue homogenates, extracts, and purified enzymes were studied. Using various radioactive substrates, radioactive CO_2 was collected as a measure of carbohydrate oxidation. Where indicated, standard enzymatic assays and biochemical analyses were performed.

Major Findings:

Glucose oxidation - Using C_1 and uniformly labeled glucose C^{14} as substrate, rat liver slices and crude homogenates showed significant stimulation of $C^{14}O_2$ production when incubated in the presence of various steroids in concentrates as low as 5×10^{-6} M. Addition of large amounts of hexokinase served only to enhance the effects and similar stimulation was observed with G-6-P as substrate. Furthermore direct assays of hexokinase failed to reveal any steroid stimulation. Such experiments were done using conditions under which glucose was presumed to be oxidized almost exclusively via the TPN specific hexosemonophosphate shunt.

Part B included Yes [ ] No [X]

Finally, working with ultracentrifugate of rat liver it was possible to show that stimulation of CO_2 production from Glucose-6-phosphate resulted from steroid A ring reduction by the mechanism of TPN production. (Steroid reduction is TPNH specific.) Steps in proof included: (1) abolishing the effect with tremendous excesses of TPN, (2) correlation of individual steroid reductases with the degree of stimulatory effect shown by the steroid; (3) stoichiometry of the overall reactions. Furthermore, the hexose-monophosphate shunt was not stimulated in tissue lacking steroid reductases.

Lactate and Pyruvate Oxidation - $C^{14}O_2$ production from C_2 labeled (and to a lesser extent C_1 labeled) pyruvate and lactate by rat liver homogenate was significantly enhanced by cortisone and testosterone in concentrations as low as 10^{-7} M. Addition of TPN was essential for such an effect and ATP in high concentration would abolish the effect. Interestingly, liver homogenates from fasted animals either showed no effect by steroid or showed inhibition. Preliminary screening with a large group of steroids does not show good correlation with steroid reduction activity, or the potential action of the steroid as a transhydrogenase co-factor. Further work is actively in progress.

Significance to NIAMD Research: Such data as sought in this project offer the possibility of elucidating the mechanism of action of corticosteroids and the role they play in regulating the rate of certain basic enzymatic processes of considerable biological importance.

Proposed Course of Project: We will continue to explore the relative effects of different steroids on overall metabolic fate of lactate and pyruvate, and ultimately on specific enzymatic steps. Results will be correlated with physiologic activity.

Serial No. NIAMD- 122-C
1. Clinical Investigation
2. Arthritis and Rheumatism
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: The Metabolism of Adrenocortical Steroids

Principal Investigator: Ralph E. Peterson, M.D.

Other Investigators: Bernard Kliman, M.D.
Saul Rosen, M.D.
William Zorbach, M.D.

Cooperating Units: National Heart Institute
(James Davis, M.D.)

Man Years (calendar year 1958):
Total: 5
Professional: 2-1/3
Other: 2-2/3

Project Description:

Objectives: To determine the rate of metabolism of aldosterone, factors regulating its secretion and the binding of adrenal cortical steroids to plasma proteins.

Methods Employed:

Double Isotope Derivative Method for Assay of Adrenocortical Steroids. Because of the need for sensitive and specific methods of assay of steroids in biological extracts further work was carried out on extending and improving these physical isotopic methods of assay. The availability of isotopes of higher specific acticity made possible improvements in the assay of urine and adrenal vein aldosterone concentrations. Also it was possible to assay for aldosterone in human peripheral plasma with a double isotope dilution derivative method using tritium-labeled aldosterone. Normal plasma aldosterone level was found to range from 0.04 to 0.08 µg./100 ml. and this level may increase two- to three-fold after sodium restriction.

Major Findings:

Metabolism of Aldosterone. The use of another modification of the double isotope derivative methods made possible a measure of the rate of secretion of aldosterone in normal subjects and certain patients. Secretion rates of 350, 360 and 400 µg. of aldosterone per day were found in normal

subjects. On a low sodium diet the secretion rate increased two- to three-fold. A patient with hyperaldosteronism had a secretion rate of 640 μg. per day on an ad lib diet. Patients with cirrhosis maintained on a low sodium diet had increased secretion rates indicating that the secondary aldosteronism represented overproduction of aldosterone. In patients with cirrhosis the biologic half time of aldosterone was not greatly different from the half time values of 30 to 60 minutes found in normals. In one normal subject the rapidly exchangeable miscible pool of aldosterone was calculated to be 10 μg.

Mechanism of Secretion of Aldosterone. In collaboration with NEI scientists studies were carried out on the factors regulating the secretion of aldosterone in dogs. Most of these studies involved measurements of adrenal secretion rate of aldosterone in dogs with thoracic caval constriction and ascites associated with hyperaldosteronemia. In one study designed to evaluate the relation of the vascular volume it was possible to demonstrate that increased aldosterone secretion following acute constriction of the inferior vena cava above or below the hepatic veins occurred irrespective of changes in the vascular volume. In another study the acute effects of hypophysectomy and subsequent diencephalic lesions on aldosterone secretion in dogs with chronic ascites was made. These acute observations showed the striking dependence of both aldosterone and corticosterone secretion on intact pituitary function. The evidence of the relation of the diencephalon to aldosterone secretion was inconclusive, but an intact middle and posterior hypothalamus was not essential for normal or elevated rates of aldosterone secretion. In another set of experiments an attempt was made to evaluate the presence of a circulating humoral agent that stimulates the adrenal cortex to secrete aldosterone independent of corticotropin. This was done by the use of cross-circulation between the caval-constricted dog and a normal-recipient dog with an isolated adrenal vein pouch. By studying the aldosterone secretion rate from the adrenal vein pouch during the cross-circulation it was possible to clearly demonstrate the presence of a trophic hormone for aldosterone that was not corticotropin, viz. increased rate of secretion of aldosterone with no associated increased rate of secretion of cortisol or corticosterone.

Protein Steroid Binding. There is considerable evidence that the adrenocortical steroids are transported in the circulation bound to proteins. It therefore was of interest

to determine the fraction of the steroid that was protein bound and the fraction that was circulating free since it may be that only the latter fraction possesses biologic activity. Studies were initiated using both ultrafiltration and dialysis equilibration techniques. The preliminary data have served to confirm the data as obtained by other investigators, namely in studies carried out at 4° C more than 99% of the steroid is protein bound, and at 37° C approximately 96 to 97% of the steroid is protein bound at physiologic concentrations of cortisol (5 to 25 μg.%). At greater than physiologic concentrations only 70 to 80% of the steroid is protein bound. Following estrogen therapy there is an increase in the concentration of the protein-bound steroid so that at plasma cortisol levels of up to 60 μg.% more than 95% of the cortisol remains protein bound. Perhaps this observation explains the lack of biologic effectiveness of the elevated levels of cortisol seen following estrogen therapy.

Part B: Honors, Awards, and Publications

Publications:

1. Peterson, R. E.: The Miscible Pool and Turnover Rate of Adrenocortical Steroids in Man. Laurentian Hormone Conference, 1958 (in press).

2. Peterson, R. E.: The influence of the thyroid on adrenal cortical function. J. Clin. Invest. 37:736-743, 1958.

3. Peterson, R. E., Black, R. L., and Bunim, J. J.: Salicylates and adrenocortical function in Man. Arth. & Rheum. 1:29-37, 1958.

4. Seven, M. J. and Peterson, R. E.: Studies on the *in vivo* stability of an iron chelate. Proc. Soc. Exper. Biol. & Med. 97:382-384, 1958.

5. Ebaugh, F. G., Jr., Clemons, T., Jr., Rodman, G., and Peterson, R. E.: Quantitative measurement of gastrointestinal blood loss. I. The use of radioactive Cr51 in patients with gastrointestinal hemorrhage. Am. J. Med. 25:169-181, 1958.

Honors and Awards:

Dr. Ralph Peterson was offered the position of Associate Professor of Medicine at Cornell University College of Medicine beginning September 1958. Dr. Peterson accepted this post and resigned from NIH.

Serial No. NIAMD - 1958
1. Clinical Investigations
2. Arthritis and Rheumatism
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Trial of New Anti-Rheumatic Drugs

Principal Investigator: Dr. Joseph J. Bunim

Other Investigators: Drs. Roger L. Black, Kurt J. Bloch, John R. David, George E. Ehrlich, and Ralph E. Peterson

Cooperating Units: There were no cooperating units elsewhere in the Public Health Service

Man Years (calendar year 1958):
Total: 3-1/2
Professional: 2-5/6
Other: 2/3

Project Description:

Objectives:

1) The newer anti-rheumatic compounds have been evaluated with several objectives in view.

a. To assess relative therapeutic potency

b. To detect early the undesirable side effects, if any

c. To delineate which families of compounds show the most promise as therapeutic agents, and hence to direct further development in appropriate areas.

Methods Employed: The evaluation of drugs has been carried out on an in-patient group at the Clinical Center. In addition to this, a Rheumatic Diseases service has been established on the Georgetown Medical Service at D.C. General Hospital, where new therapeutic compounds are evaluated in out-patients with rheumatoid arthritis. Details of the studies in regard to anti-inflammatory effect, side effect evaluation, and metabolic and hormonal observations have been published.

Part B included Yes [X] No [ ]

Patient Material:

Major Findings: The following compounds have been studied:

1) 16α methyl 9α fluoroprednisolone (MK-125)
2) 16β methyl 9α fluoroprednisolone (MK-149)
3) 16α methyl prednisone (MK-109)
4) 16β methyl prednisone (MK-129)
5) 6α fluoro hydrocortisone (U-8107)
6) 6 methyl, 21 deoxy, 9α fluoroprednisolone (U-8614)

Dexamethasone (16α methyl 9α fluoroprednisolone) has now been evaluated in a total of 23 patients -- 18 in-patients and 5 out-patients. One patient died. On maintenance dosage of 0.5 to 4 mgms. daily, supplemented with buffered or enteric coated salicylates in dosage of 2.4 to 4.8 gms. daily in 9 cases, 22 patients achieved the following functional state:

Class 1 --- 10 patients
Class 2 --- 7 "
Class 3 --- 3 "
Class 4 --- 0 "

This compound has been found to be a potent suppressor of corticotropin secretion. A single dose of 1.0 mg. was sufficient to reduce plasma hydrocortisone level to zero. Neither sodium retention nor potassium loss occurred on doses up to 6 mgms. daily. Aldosterone production and responsiveness remained adequate in a normal subject given 6 mgms. daily, while on a markedly reduced sodium intake.

The most troublesome side-effect has been a marked increase in appetite, 13 patients having gained from 3 to 17 kg. in weight over a 3 to 6 month period. Twenty have developed Cushingoid facies.

These patients have been observed closely for gastrointestinal disturbances. Four have had slight, epigastric distress which has not persisted. One of these patients with a history of a previous ulcer underwent gastrectomy. Repeated roentgenological examinations of the gastro-intestinal tract failed to uncover ulceration of the stomach or duodenum in any case. Chemical tests for occult blood in stools were persistently negative in 12 cases and only occasionally positive in 3.

Glucose utilization tests have been done repeatedly in 21 of the 22 patients. In none has the utilization rate become abnormal, when pretreatment tests were found normal. In 5 patients, previously abnormal while receiving maintenance doses of prednisone, the rate has improved and in 2 it has returned to normal levels.

In two patients with active rheumatoid arthritis, both urinary and fecal calcium excretion were significantly increased when the daily dose of dexamethasone was increased to 6.0 mg. This did not occur on 2.0 mg. daily. (The maximum therapeutic dose in rheumatoid arthritis is 4.0 mg. daily).

The 16β methyl analogues of MK-109 (16α methyl prednisone, MK-139) and MK-125 (16α methyl 9α fluoro prednisolone, MK-149), were evaluated. Both of these congeners were found to have anti-inflammatory properties. Potency of MK-139, was about the same as that of the alpha analogue in 2 patients. MK-149 was found less potent than its corresponding alpha steroid.

6α fluoro hydrocortisone (U-8107) was evaluated in 2 patients. No sodium retention was observed on doses of 30 and 50 mgms. of this analogue. Anti-rheumatic potency was approximately 1/3 that of prednisone.

6 methyl, 21 desoxy, 9α fluoro prednisolone (U-8614) was administered in a short term trial to three rheumatoid arthritis patients. This compound was found to have no adverse mineralocorticoid effects in a 40 to 100 mgm. dosage range. Its anti-inflammatory potency was 1/4 that of prednisone.

Significance to NIAMD Research: This study continues to provide experience with new therapeutic compounds, leading to improved management of rheumatic disease patients. Dexamethasone is the most potent anti-rheumatic steroid thus far synthesized. In therapeutic doses it is relatively free of sodium retention or potassium loss in almost all cases. In the small group tested it has not caused detectable impairment of carbohydrate metabolism. It was found effective in some patients who have stopped responding to prednisone therapy. However, dexamethasone is not free of certain major undesirable side-effects such as fractures of vertebral bodies and (in a group of rheumatoid arthritis patients observed by other workers) peptic ulcers. Thus far (9 months of trial) other major side-effects have not appeared, such as mental changes, psychosis, hypertension and steroid diabetes.

PHS-NIH
Individual Project Report
Calendar Year 1958

Part B: Honors, awards, and publications

Publications:

1) Bunim, J. J., Black, R. L., Ratwak, L., Peterson, E. E., and Whedon, G. D.: Studies on dexamethasone, a new synthetic steroid, in rheumatoid arthritis -- a preliminary report. Arth. & Rheum. 1: 313-331, 1958.

2) Bunim, J. J.: Critical evaluation of synthetic steroids in the treatment of rheumatoid arthritis. Arch. Internmed. Rheum. 1: 464-482, 1958.

3) Black, R. L., and Bunim, J. J.: Progressive systemic sclerosis (scleroderma) and dermatomyositis. Med. Clin. of North America. Nov.:1653-1662, 1958.

4) Ference, C., Markowitz, M., and Bunim, J. J.: The effect of large doses of prednisone on acute rheumatoid factor: Observations on the treatment of 17 patients with carditis with a two-year followup. Am. Jl. Dis. Child. (in press).

5) Moderator - Bunim, J. J.; Discussants - Federman, D. D., Black, R. L., Schmid, R., Sokoloff, L., and Shurley, J.: Factitious diseases: Clinical staff conference at the National Institutes of Health. Ann. Int. Med. 48: 1328-1341, 1958.

6) Shulman, L. E., and Bunim, J. J.: Disorders of the joints. In Harrison, T. R. (ed.): Principles of Internal Medicine. New York, McGraw-Hill Book Co., 1958, Third Ed. 1712-1733.

7) Kellgren, J. H. (ed.): Transactions of the First International Conference on Population Studies in Rheumatoid Arthritis, McGregor and Werner, Inc., Washington, D. C., 1958.

Honors and Awards:

Dr. Joseph J. Bunim was elected President of the American Rheumatism Association and appointed Co-chairman of the Pan American Congress.

Serial No. 126-C
Clinical Investigations
Arthritis & Rheumatism Br.
Bethesda, Maryland

PHS - NIH
Individual Project Report
Calendar Year 1958

Part A

PROJECT TITLE: Studies on Alcaptonuria and Ochronotic Arthritis in Man and Animals

PRINCIPAL INVESTIGATOR: Bert N. La Du

OTHER INVESTIGATORS: J. E. Seegmiller, V. Zannoni, L. Lester, and W. O'Brien

COOPERATING UNITS: None

MAN YEARS (Calendar year 1958):
Total: 4-1/2
Professional: 3-1/2
Other: 1

PROJECT DESCRIPTION:

Our aims in studying patients with alcaptonuria have been several: (1) to determine the exact nature of the metabolic defect in this condition; (2) to study the hereditary pattern of this disease, and, if possible, to develop a test which will detect the heterozygous state in relatives of alcaptonurics carrying the trait; (3) to study the formation and deposition of the pigment derived from homogentisic acid and to determine how it produces the pathological changes in the connective tissues, particularly the joints; (4) to study the cause of the arthritis nearly always associated with this condition, and (5) to attempt various means of treatment of this metabolic disease.

Clinical Studies:

Nature of defect - Quantitative assay of the enzymes involved in tyrosine degradation was made in kidney homogenates (autopsy samples) of alcaptonuric and non-alcaptonuric patients. Alcaptonuric kidney was found to differ from the normal only in having no detectable homogentisic acid oxidase activity; this enzyme was easily detected in the non-alcaptonuric kidney samples. We can now extend our earlier observations on the nature of the defect in alcaptonuria to include the lack of homogentisic acid oxidase activity in the kidney as well as the liver.

Detection of the carrier trait - The pattern of inheritance of alcaptonuria is being re-investigated by review of all of the

published family pedigrees. Our preliminary analysis is that simple recessive transmission seems to explain all of the well documented cases. Evidence favoring direct transmission in some families is not convincing.

Attempts to detect a biochemical alteration in homogentisic acid metabolism by oral tolerance tests in relatives of alcaptonuric patients have so far shown no difference from normal individuals.

Animal Studies:

1. Experimental alcaptonuria and ochronotic arthritis - Several inhibitors of homogentisic acid oxidase are being tested as inducers of experimental alcaptonuria. In addition, homogentisic acid and a pigment derived chemically from homogentisic acid are being injected into guinea pigs to produce ochronosis and ochronotic arthritis.

2. Enzymatic synthesis of homogentisic acid - Detailed studies of the enzyme system of liver-forming homogentisic acid from parahydroxyphenylpyruvic acid (the keto acid of tyrosine) are being continued. We have found that vitamin C not only prevents an inactivation of this enzyme by its own substrate, but it also affects the renal reabsorption of p-hydroxyphenylpyruvic acid. The observed excretion of p-hydroxyphenylpyruvic acid by scorbutic guinea pigs is therefore partly due to a failure to metabolize this acid, and in part to its deficient reabsorption in the kidney. The possibility that vitamin C has a similar effect in the kidney on the reabsorption of other acids [illegible] is to be investigated.

Chemical Studies:

1. Method for the estimation of homogentisic acid in plasma - In the course of these investigations, a specific enzymatic method to measure small amounts of homogentisic acid in plasma was developed (J.E.S.). This method will be useful not only as a more specific diagnostic test to differentiate homogentisic acid from other reducing compounds in plasma and urine, but also permits the analyses of blood samples during tolerance tests with homogentisic acid.

2. Method for measuring plasma phenylalanine - A specific micro method has been developed to measure phenylalanine enzymatically in plasma which we feel is superior to any other methods now available. This method will be of value in diagnosing phenylketonuria (where plasma phenylalanine is elevated) and in the determination of plasma phenylalanine levels in tolerance tests employed to detect the phenylketonuria trait.

3. Pigment formation from homogentisic acid - The production of a melanin-like pigment from pure homogentisic acid is being studied by chemical methods. We have separated some intermediary compounds in this reaction by paper chromatography to determine the sequence of the

steps involved. We hope to test some of these intermediates in guinea pigs to see whether they will cause the production of ochronotic pigments in the connective tissues and induce arthritic changes in the joints (experimental ochronotic arthritis).

Part B included: Yes

Serial No. 124-C

PHS - NIH
Individual Project Report
Calendar Year 1958

Part B: Honors, Awards, and Publications

PUBLICATIONS:

1. La Du, B. N., Zannoni, V., Laster, L., and Seegmiller, J. E. The nature of the defect in tyrosine metabolism in alcaptonuria. J. Biol. Chem., 230: 251-260, 1958.

2. La Du, B. N., Laster, L., Seegmiller, J. E., and Zannoni, V. Alcaptonuria and ochronotic arthritis. Bulletin on Rheumatic Diseases, 8:163-164, 1958.

3. La Du, B. N. Alcaptonuria and ochronosis. Chapter in textbook, Biochemistry of Molecular Diseases, edited by J. Stanbury, J. B. Wyngaarden and D. Fredrickson. New York, McGraw-Hill. In press.

Serial No. 125-C
Clinical Investigations
Arthritis & Rheumatism Br.
Bethesda, Maryland

PHS - NIH
Individual Project Report
Calendar Year 1958

Part A

PROJECT TITLE: Metabolic and Therapeutic Studies of Gouty Arthritis and Hyperuricemia

PRINCIPAL INVESTIGATOR: J. E. Seegmiller

OTHER INVESTIGATORS: Leonard Laster, Arthur Grayzel, Joseph McGuire, and Lois Liddle

COOPERATING UNITS: None

MAN YEARS (Calendar year 1958):
Total: 2-1/2
Professional: 2
Other: 1/2

PROJECT DESCRIPTION:

An evaluation of therapeutic agents for management of problem patients with gouty arthritis has been continued. The new uricosuric agent, sulfinpyrazone (G-28315) reported in last year's work has been further studied in problem cases of tophaceous gout. In addition, studies have been made of the uricosuric action of zoxazolamine (Flexin) which has been in use for several years as a muscle relaxant and has been found by NIH grantees to have a very potent uricosuric activity equal to that of sulfinpyrazone. Even more important, the uricosuric activity of these two drugs is synergistic, in that the two drugs together produce a greater uric acid excretion than either drug alone. Such findings greatly extend the range of therapeutic action available to the physician in managing problem cases of gouty arthritis which have not responded to conventional treatment with probenecid, or who cannot take probenecid because of objectionable side effects. Although salicylates in low doses tend to suppress somewhat the uricosuric activity of zoxazolamine, preliminary studies indicate that at high dosages of salicylates, the uricosuric activity is additive to that of zoxazolamine. This is in contrast to the antagonistic action between large doses of salicylates and either probenecid or sulfinpyrazone, where salicylates negate the uricosuric action of these drugs in most patients.

Part B included: No

Serial No. 126-C
Clinical Investigations
Arthritis & Rheumatism Br.
Bethesda, Maryland

PHS - NIH
Individual Project Report
Calendar Year 1958

Part A

PROJECT TITLE: Metabolism of Uric Acid Ribonucleoside in Mammalian Tissues

PRINCIPAL INVESTIGATOR: Leonard Laster

OTHER INVESTIGATORS: Alberta Blair

COOPERATING UNITS: None

MAN YEARS (Calendar year 1958)
Total: 1
Professional: 1/2
Other: 1/2

PROJECT DESCRIPTION:

In man and other mammals uric acid is produced by the oxidation of xanthine in the presence of xanthine oxidase. The detection of an alternate enzyme-catalyzed reaction in which uric acid arises was described in the 1957 report on this project. Since then the nature of the reaction has been defined more precisely, and many properties of the enzyme have been studied. Of potential interest in relation to the pathologic physiology of gout is the observation that the alkaloid colchicine, a specific remedy for the acute arthritis of this disease, is a competitive inhibitor of the enzyme.

The enzyme, which was named uric acid ribonucleoside phosphorylase, catalyzes the reaction:

(1) Uric acid ribonucleoside + orthophosphate $\rightleftharpoons$ uric acid + ribose-1-phosphate.

It was purified 100-fold from mucosa of dog small intestine and the final preparation failed to cleave the ribonucleosides of adenine, hypoxanthine, guanine and xanthine. Although uric acid ribonucleoside phosphorylase and inosine phosphorylase were clearly

shown to be separable, thymidine phosphorylase activity persisted in constant proportion to the uric acid ribonucleoside enzyme at each step of its purification. It has not yet been determined whether the two enzyme activities are distinct or identical.

The reversal of reaction (1) could not be demonstrated with certainty despite the use of a wide variety of reaction conditions. If the reaction is reversible, at the equilibrium point ribonucleoside cleavage is favored. With inosine and its analogous enzyme, synthesis is favored.

Uric acid ribonucleoside phosphorylase is widely distributed among the tissues of rabbits and is most abundant in bone marrow and intestinal mucosa, two tissues undergoing active cell division. He La cells, also actively dividing cells, contain readily detectable levels of the enzyme. It has been found in tissues from beef, dog, guinea pig, hamster and man.

The purified dog preparation was inhibited by p-chloromercuribenzoate in low concentration, and this inhibition could be prevented by the presence of the sulfhydryl groups of the enzyme protein. Many other inhibitors of the enzyme were found including colchicine, a sulfoxide derivative of phenylbutazone, and phenylbutazone, listed in decreasing order of inhibitory capacity. Benemid and sodium salicylate failed to inhibit. By use of Lineweaver-Burk plots it was found that colchicine is a competitive inhibitor of uric acid ribonucleoside phosphorylase, and that the enzyme has the same affinity for colchicine as it does for uric acid ribonucleoside. Under conditions permitting 90% inhibition of uric acid ribonucleoside cleavage by colchicine, inosine phosphorylase activity was inhibited only 4%.

The results of the studies in this project suggest that in mammals the oxidation of xanthine is not the sole pathway for the production of uric acid. Whether the alternate pathway described above plays an important role in purine metabolism cannot be decided until the metabolic origin of uric acid ribonucleoside is elucidated. This is an important future objective in this project. Although the occurrence of uric acid ribonucleoside in human erythrocytes was reported in 1922, this finding could not be confirmed by two subsequent investigators. We attempted to detect this ribonucleoside in erythrocytes from normal subjects and one gouty patient and were unable to do so. It appears, therefore, that if the ribonucleoside occurs in human tissues, it probably occurs only in trace amounts, not unlike many other

intermediate compounds in metabolic sequences. It may be possible to demonstrate that in animals colchicine poisoning will cause the ribonucleoside to appear in the urine or accumulate in the blood and if this could be shown, then one could study urine and blood from gouty patients receiving colchicine to the point of toxicity. This is a second proposed extension of the project.

Another avenue of investigation suggested by this work concerns the question of whether inhibition of uric acid ribonucleoside phosphorylase is in any way involved in the therapeutic action of colchicine in acute gouty arthritis. One approach to this problem is to study whether other in vitro inhibitors of the enzyme have a beneficial effect on acute gouty arthritis. Propylthiouracil is a more potent inhibitor of the dog enzyme than is colchicine. In a preliminary study relatively low doses of this compound were administered to a patient who was in the midst of an acute attack of gouty arthritis. No beneficial effect was noted, but further study is in order before any conclusions can be drawn.

Work on this project was stopped in July, 1958, but it is hoped that it can be resumed in July, 1959.

Part B included: No

Serial No. 127-C
Clinical Investigations
Arthritis & Rheumatism Br.
Bethesda, Maryland

PHS - NIH
Individual Project Report
Calendar Year 1958

Part A

PROJECT TITLE: The Abnormalities of Purine Metabolism Associated with Gout

PRINCIPAL INVESTIGATOR: J. E. Seegmiller

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS (Calendar year 1958)
Total: 2
Professional: 1/2
Other: 1-1/2

PROJECT DESCRIPTION:

Further studies of the metabolic origin and disposition of uric acid in gouty patients have been obtained by administering isotopically labeled precursors of uric acid along with labeled uric acid itself to patients with gout and hyperuricemia and determining the pattern and extent of incorporation of the isotope into urinary uric acid. In previous studies of Dr. Wyngaarden, six gouty subjects and one subject with essential hyperuricemia were reported to show consistently greater incorporation of glycine-1-C^{14} into urinary uric acid than did three non-gouty subjects. These findings were interpreted as evidence that the primary metabolic defect in gout is excessive production of uric acid. We have been able to show that although a substantial number of gouty patients do show incorporation of excessive amounts of this isotope into urinary uric acid, there are others who show an incorporation indistinguishable from that of normal non-gouty subjects, so that such excessive incorporation cannot be a defect characteristic of all gouty patients.

Pharmacological agents which might result in a suppression of the excessive synthesis of uric acid in gouty subjects have been investigated. Of a number of such agents tested, one that has shown to be effective is 6-diazo-5-L-norleucine (DON), a glutamine antagonist, which has been shown by *in vitro* studies of other workers to inhibit a specific enzyme that uses glutamine in purine biosynthesis. One patient with severe tophaceous gout and polycythemia

rubra vera showed evidence of decreased uric acid synthesis as evidenced by a lower incorporation of glycine-1-C^{14} into urinary uric acid while receiving DON over a 5-day period. A second patient with excessive uric acid production and urate renal lithiasis showed a fall in his serum urate level from 8.6 mg.% to 6.5 mg.% and a drop in his daily uric acid excretion from 1369 mg./day to 730 mg./day in a 5-day course of therapy with DON. Such preliminary results give promise that pharmacological agents may be effective in controlling the excessive uric acid production seen in some gouty patients.

Part B included: Yes

Serial No. 127-C

PHS - NIH
Individual Project Report
Calendar Year 1958

Part B: Honors, Awards, and Publications

1. Seegmiller, J. E., and Laster, L. The Metabolic Origin of Uric Acid. Progress in Arthritis, edited by Drs. John Talbott and Maxwell Lockie, published by Grune & Stratton, Inc., New York, 1958.

2. Seegmiller, J. E., Laster, L., and Liddle, L. Failure to Detect Consistent Overincorporation of Glycine-1-C^{14} into Uric Acid in Primary Gout. Metabolism, 7:376, 1958.

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

ANNUAL PROJECT REPORT

CALENDAR YEAR 1958

Summary Sheet

CLINICAL ENDOCRINOLOGY BRANCH

Estimated Obligations for FY 1959

Total - $409,954
Direct - $249,600
Reimbursement - $160,354

Project Numbers 128-C thru 139-C Included

Serial No. 128-C
1. Clinical Investigations
2. Clinical Endocrinology Branch
3. Bethesda

FHS-NIH
Individual Project Report
Calender Year 1958

Part A:

Project Title: Humoral Antagonists to Insulin

Principal Investigator: James B. Field, M. D.

Other Investigators: Arnold Weinberg, M. D. and Phyllis Johnson

Cooperating Units:

Man Years (calendar year 1958): Patient Days (calendar year 1958):
Total: 2-1/6
Professional: 1-1/2
Other: 2/3

Project Description:

Utilizing the glucose uptake by the isolated rat hemidiaphragm as a measure of insulin activity, a test system was devised so that the addition of 10 milliunits of insulin to 1 ml. of normal plasma produced an increase in glucose uptake of 2.7% mg./gm. of muscle. When plasma from four acromegalic patients with diabetes was tested, in three of them it was not possible to recover the effect of the added insulin. Two of four new untreated diabetic patients also had evidence of an insulin antagonist in their plasma. Although these patients had glycosuria and ketonuria, they were not in diabetic acidosis. Three days later, when the diabetes had been controlled with insulin, it was no longer possible to demonstrate antagonist. Three of four patients with uncontrolled diabetes secondary to an infection also had evidence of an insulin antagonist. In one patient tested, the antagonist was still active even though the diaphragm was exposed to plasma and insulin separately. This is very similar to the behavior of the insulin antagonist which we have previously described during diabetic acidosis.

Studies were also carried out on plasma from several patients with chronic insulin resistance. Again it was possible to demonstrate an insulin antagonist, but in this situation it was necessary to have diaphragm, plasma and insulin all present simultaneously to produce antagonism. The amount of antagonist was found to decrease concomittant with the successful treatment of the insulin resistance with prednisone.

Part B included Yes [X] No [ ]

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Field, J. B.: Observations concerning a humoral insulin antagonist during diabetic ketosis. Diabetes 7:433-438, 1958.

Field, J. B. and Rigby, B.: Circulating insulin antagonists in diabetes mellitus. J. Endocrin. In press.

Honors and Awards relating to this project:

Dr. James Field was awarded the 1958 Lilly Award of The American Diabetes Association for outstanding research work in the field of diabetes by an investigator under the age of 40.

Serial No. 139-C
1. Clinical Investigations
2. Clinical Endocrinology Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A:

Project Title: Studies in Carbohydrate Metabolism

Principal Investigator: Stanton Segal, M. D.

Other Investigator: Alberta Blair

Cooperating Units:

Man Years (calendar year 1958): Patient Days (calendar year 1958)
Total: 1-2/3
Professional: 1
Other: 2/3

Project Description:

During the period July to December 1958 the following projects have been under investigation:

The Effects of Progesterone on Galactose Metabolism- Based on the Topper and Pesch observation that progesterone stimulates galactose oxidation to CO_2 in vitro, the effect of progesterone on the production of cataracts in rats on high galactose diets has been studied. Preliminary results suggest that progesterone protects rats from getting cataracts from galactose. Another phase has been to study progesterone effects in a galactosemic individual who cannot oxidize galactose to CO_2. In one such patient, progesterone caused a conversion of C^{14} labeled galactose to CO_2. These above observations may lead to therapeutic usefulness of progesterone in patients with galactosemia.

Effects of Salicylate on Glucose Metabolism- Recent observations have suggested salicylate is effective in treating diabetes mellitus. We have studied in vitro effects of salicylate on isolated rat diaphragm and epididymal fat pad. It has been found that salicylate has profound effects on glucose metabolism in rat diaphragm. Salicylate markedly stimulates glucose oxidation to CO_2 but has no effect on glucose uptake and does not modify responsiveness to insulin. Salicylate markedly inhibits the enzyme muscle phosphorylase. No results have been obtained to explain the therapeutic effect of salicylate in diabetes.

The Metabolism of Variously C^{14} labeled Glucose in Man- Rates of $C^{14}O_2$ production and turnover of blood glucose is being ascertained in normals, diabetics and various endocrine diseases. Results are too preliminary for comment.

Part B included Yes [X] No [ ]

Part B: Honors, Awards, and Publications

Publications other than abstracts from this Project:

Segal, S., Foley, J. F.: Metabolism of C^{14} Labeled Pentoses in Man. J. Clin. Invest. 38: Feb. 1959.

Segal, S. and Foley, J.: The Metabolism of D-Ribose in Man. J. Clin. Investig. 37:719, 1958.

Segal, S.: Pentose Metabolism in Man. Diabetes 7:147, 1958.

Serial No. 130-C
1. Clinical Investigations
2. Clinical Endocrinology Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A:

Project Title: Thyroxine-binding Proteins of Serum

Principal Investigator: Jacob Robbins, M. D.

Other Investigators: J. E. Rall, M.D., D. Jermany and V. Tredway

Cooperating Units:

Man Years (calender year 1958): Patient Days (calendar year 1958):
Total: 2-5/6
Professional: 5/6
Other: 2

Project Description:

The work reported in previous annual reports on the influence of the sex hormones on the thyroxine-binding alpha globulin (TBP) has been published. These studies demonstrated a considerable increase in the thyroxine-binding capacity of TBP during pregnancy - apparently a result of increased estrogen secretion - and conversely, a decrease of binding capacity in patients given testosterone. The studies suggest that the sex hormones have a primary effect on the concentration of TBP in blood. The effects which these hormones have on thyroid gland function and thyroid hormone metabolism appear to be secondary to this protein change. Studies are continuing on the influence of other substances - e.g. adrenal steroids and testosterone analogues (e.g. 17-methyl-19 nortestosterone) - on TBP. In these binding studies, the technique of paper electrophoresis in barbital buffer has been employed. Since recent studies have shown that other buffers give qualitatively different patterns of thyroxine-protein interaction, the validity of the TBP binding capacity measurement in barbital buffer has been checked. The values are essentially the same in barbital buffer and in bicarbonate buffer of low ionic strength. Electrophoresis in bicarbonate buffer, as well as in a number of other buffers, gives a thyroxine-zone migrating faster than albumin. This zone is present to a much smaller degree in barbital buffer. The reason for this difference is currently under study.

Part B included Yes [X] No [ ]

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Robbins, J. and Nelson, J. M.: Thyroxine-binding by Serum Protein in Pregnancy and in the Newborn. J. Clin. Investig. 37:153-159, 1958.

Federman, D., Robbins, J. and Hall, J. E.: Effects of Methyl Testosterone on Thyroid Function, Thyroxine Metabolism and Thyroxine-binding Protein. J. Clin. Investig. 37:1024-1030, 1958.

Federman, D., Robbins, J. and Hall, J. E.: Some Observations on Cretinism and its Treatment. N. E. J. of Med. 259:610-615, 1958.

Federman, D. D.: Studies on Cretinism. G. P. 18:114, 1958.

Serial No. 131-C
1. Clinical Investigations
2. Clinical Endocrinology Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A:

Project Title: Mammalian Iodoproteins

Principal Investigator: Jacob Robbins, M. D.

Other Investigators: Mitchell T. Rabkin, M.D., Susan Barbella and Helen Postman

Cooperating Units: NCI - 906

Man Years (calendar year 1958):
Total: 2-1/3
Professional: 1-1/2
Other: 5/6

Patient Days (calendar year 1958):

Project Description:

The study of iodine-containing thyroid proteins has continued both in rat thyroid tumors and in human thyroid tissue obtained at surgery. In vivo and in vitro labeling techniques with radioiodine have been employed. At least two classes of iodoproteins, heretofore unknown, have been detected and partially characterized. Both are quite different from the well known thyroglobulin molecule, which normally comprises most of the thyroid iodine. One of these iodoproteins is soluble in isotonic sodium chloride, as is thyroglobulin, but differs in its salt solubility, ultracentrifugal, and electrophoretic properties, as well as in its content of iodinated amino acids. This iodoprotein resembles closely the iodoprotein found in blood in certain thyroid diseases and probably is its precursor. Interestingly enough, the species difference noted in this thyroid protein between rat and man is also seen in the abnormal blood iodoprotein in rats and patients with thyroid carcinoma.

The second class of iodoprotein remains with the particulate fraction of the thyroid tissue after homogenization. Further fractionation has disclosed that most, if not all, of this substance is associated with cell nuclei, and remains insoluble after extraction of nucleic acids and histones from the nuclei. Treatment of the residue with 0.1 N Sodium hydroxide results in solubilization of the iodoprotein, which then appears to be a relatively small molecule. Column chromatography has indicated that this material may be made up of several components.

Part B Included Yes [X] No [ ]

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Robbins, J., Wolff, J. and Rall, J. E.: Iodoproteins in Thyroid Tissue and Blood of Rats with a Transplantable Thyroid Tumor. Endocrinology. In press.

Robbins, J., Wolff, J. and Rall, J. E.: Iodoproteins in Normal and Abnormal Human Thyroid Tissue and in Normal Sheep Thyroid. Endocrinology. In Press.

Small quantities of the soluble and particulate iodoproteins have been found in normal thyroid glands, but they are present in increased amount in certain thyroid diseases. The soluble iodoprotein is somewhat increased in some human thyroid adenomas. The particulate iodoprotein is greatly elevated in the rat thyroid tumors. A similar elevation was found in one human thyroid carcinoma.

The physiological importance of these iodoproteins is yet to be determined. Their discovery opens a new aspect of thyroid biochemistry which already appears to be implicated in thyroid disease. Further studies on the particulate iodoprotein should, in addition, provide information concerning the so-called "residual protein" of cell nuclei.

Serial No. 122-C
1. Clinical Investigations
2. Clinical Endocrinology Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A:

Project Title: Studies on Iodide Concentration.

Principal Investigator: Jan Wolff, M. D.

Other Investigators: John Maurey and J. E. Rall, M.D.

Cooperating Units: NCI - 906

Man Years (calendar year 1958): Patient Days (calendar year 19[illegible])
Total: 1-5/6
Professional: 5/6
Other: 1

Project Description:

Studies on the mechanism of iodide concentration by the thyroid gland have continued. The transplantable thyroid tumor (provided us by Dr. Seymour Wollman of NCI) which is active in concentrating iodide but incapable of organifying iodine described in last year's report has been further examined. It has been found that 2,4-dinitrophenol and dicumarol *in vitro* partially inhibited iodide concentration. Triiodothyronine, thyroxine and triiodothyroacetic acid were without effect (either *in vivo* or *in vitro*). A new inhibitor of the SCN type was discovered in selenocyanate which appears to be about as potent as ClO$_4^-$. Further studies showed that various digitalis derivatives (digitoxin, strophanthidin and strophanthin K) inhibited iodide uptake at a concentration of 10^{-3}M. Although iodide concentration by the rat thyroid tumor could also be inhibited by digitoxin, concentrations of about 10^{-2}M were required. Iodide concentration by mouse salivary glands appeared to require relatively high concentrations of digitoxin (10^{-4}M). These data are of interest since digitoxin and related compounds are known to inhibit potassium flux in muscle.

Additional studies on the iodide concentrating rat thyroid tumor showed that it possessed essentially no demonstrable deiodase activity.

Part B included Yes /X/ No /___/

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Wolff, J. and Maurey, J. R.: Cardiac Glycosides and Thyroidal Iodide Transport. Nature 182: 957, 1958.

Wolff, J., Robbins, J. and Hall, J. E.: Iodide Trapping Without Organification in a Transplantable Rat Thyroid Tumor. Endocrinology 64: Jan. 1959.

Austen, F. K., Rubini, M. E. Meroney, W. H. and Wolff, J.: Salicylates and Thyroid Function. I. Depression of Thyroid Function. J. Clin. Investig. 37:1131, 1958.

Wolff, J. and Austen F. K.: Salicylates and Thyroid Function. II. The Effect on the Thyroid-Pituitary Interrelation. J. Clin. Investig. 37:1144, 1958.

Serial No. 133-C
1. Clinical Investigations
2. Clinical Endocrinology Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A:

Project Title: Studies on the Degradation of Insulin and Insulin Derivatives by Mammalian Tissues.

Principal Investigator: Glenn E. Mortimore, M. D.

Other Investigators: Frank Tietze, Ph.D. and DeWitt Stetten, Jr., M.D.

Cooperating Units: Intermediary Metabolism Section, Laboratory of Biochemistry & Metabolism, NIAMD 20

Man Years (calendar year 1958): Patient Days (calendar year 1958)
Total: 1/2
Professional: 1/2
Other:

Project Description:

Objectives - The aim of this study has been to investigate the specificity and kinetics of removal and subsequent degradation of insulin by isolated rat liver and hind-limb preparations

Methods - 1) Cyclic perfusion of rat organs with oxygenated whole blood. 2) Partition and identification of degradation products by trichloracetic acid precipitation and paper chromatography 3) Radioactivity assay.

Major findings - At essentially physiological insulin concentrations and hepatic blood flow, the cyclically perfused rat liver was observed to remove about 40% of the iodoinsulin presented to it during any single passage. Rat hind-limbs, similarly perfused removed only small amounts of iodoinsulin. Contrary to some published reports, this removal rate was not affected by tolbutamide (Orinase). Since the rate of iodoinsulin degradation was appreciably diminished with addition of native insulin, it is suggested the pathways of degradation of iodoinsulin and native insulin may be identical. Comparable "inhibition" of iodoinsulin degradation was not obtained when diluted by sulfated insulin. This is of considerable interest since the sulfated insulin appears to retain the biological potency of native insulin.

Evidence was obtained that the removal of iodine from iodoinsulin follows, rather than precedes, its proteolysis. The

Part B included Yes [X] No [ ]

addition of unlabeled mono- and diiodotyrosine completely abolished the chromatographic appearance of iodide without affecting the disappearance of iodoinsulin, suggesting that iodotyrosine is the substrate for the deiodination reaction.

Significance to NIAMD Research - The large rate of removal of iodoinsulin observed suggests that the liver may play an important role in the disposal of circulating insulin and may have significance in the amelioration of diabetes during liver failure. Since all freshly secreted insulin must pass once through liver en route to the periphery, it is difficult to reconcile this large removal with economy of hormone synthesis. It is not yet known whether insulin or its degradation products exert any direct metabolic effect on the liver.

Proposed course - The specificity of insulin capture and degradation will be extended in order to see whether slight alterations in the insulin molecule will significantly alter its susceptibility to degradation. Sulfated insulin is one such derivative which, it is hoped, will be less rapidly degraded while remaining biologically active.

The organ perfusion system applied in these studies may also be used to explore the effects of human insulin neutralizing factors and insulin-binding proteins on the capture and degradation of labeled insulin. Such studies are now being planned.

Part D Honors, Awards, and Publications

Publications other than abstracts from this Project:

Mortimore, G. E., Tietze, F., and Stetten, D., Jr. Studies on the Metabolism of Insulin-I^{131} in Isolated, Perfused Rat Liver and Hind-limb Preparations. Diabetes, in press.

Serial No. 134-C
1. Clinical Investigations
2. Clinical Endocrinology Branch
3. Bethesda

NIH-NIH

Individual Project Report
Calendar Year 1958

Part A:

Project Title: Studies of labeled-thyroxine metabolism in children and Thyroid protease.

Principal Investigator: Heskel M. Haddad, M. D.

Other Investigators: J. E. Rall, M. D.

Cooperating Units:

Man Years (calendar year 1958): Patient Days (calendar year 1958)
Total: 1-1/3
Professional: 1
Other: 1/3

Project Description:

I^{131} labeled thyroxine was injected IV. Blood samples were drawn at frequent intervals for the first 24 hours and daily thereafter for 14 days. The radioactivity in the serum was measured in a lead-shielded well with a scintillation counter. The results were plotted for each child studied on semi-logarithmic graph paper and the 1/2 time was calculated.

Other data of urine and stool excretion of the radioactive material were determined on some of the cases.

The total number of cases studied was 18 children who were chosen from the District of Columbia Children's Training School. These children were clinically euthyroid, with normal PBI, cholesterol, height age and osseous development. They were, however, mentally retarded.

In 6 children, the thyroxine binding capacity of the serum was studied. The method adopted was that described by J. Robbins

Preliminary calculations showed that I^{131}, like thyroxine, has a 1/2 time in children of 5.11 days ($\pm$ 0.07). Other data are not ready for this report.

Protease activity was determined in sheep thyroid, using a modified Anson's hemoglobin method for its assay. The aim of this study is to attempt the purification of this proteolytic enzyme and to study the various activities of this enzyme in regards to thyroglobulin breakdown and synthesis of thyroxine.

Part B included Yes [ ] No [X]

The Ancon's method was modified and adopted for assaying this enzyme. The pH range of activity was found to be between 3.3 and 5.4 with pH of maximum activity around 3.3 to 3.8. The enzyme could be separated from thyroglobulin by ultracentrifuge and salting out with PO_4 buffer (adopting a method described by J. Robbins). Results of other attempts of purification are not ready for this report.

Serial No. 135-C
1. Clinical Investigations
2. Clinical Endocrinology Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A:

Project Title: Studies of labeled protein metabolism and of thyroid physiology

Principal Investigator: Charles G. Lewallen, M. D.

Other Investigators: Louis Bunce

Cooperating Units:

Man Years (calendar year 1958): Patient Days (calendar year 1958)
Total: 2
Professional: 1
Other: 1

Project Description:

Methods - Electrophoretically homogeneous serum albumin has been prepared by starch-column electrophoresis in a pyrogen free state by special sterile techniques during electrophoresis and column elution. The protein is labelled with I^{131} and tracer studies performed in patients and in experimental animals.

NaI^{131} is injected intravenously and serial determinations of thyroid, plasma, and excreted radioactivity are performed. These studies are carried out in normal subjects and in various thyroid disorders.

Results - Iodoalbumin tracer data is analyzed with reference to a multicompartment open kinetic system. Previous studies with a commercial iodoalbumin preparation suggested the presence in the body of a compartment in very slow exchange with plasma. No convincing evidence for this compartment has been seen in tracer studies in rats where total retained label has been quantitated by whole body counting. Tracer studies in patients on high cortisone dosage have shown no striking effects other than an increase in the rate constant of excretion of iodoalbumin degradation products. It has been possible to express plasma data as a sum of exponential terms and to obtain values for the standard errors of the coefficients and exponential constants utilizing a digital computer approach worked out by Dr. Mones Berman.

Repeated I^{131} tracer studies have been performed in a goitrous cretin with an essentially isolated iodide trapping

Part B included Yes [X] No [ ]

mechanism. The trap was dischargable with CSN^- and I^-, was less active after administration of thyroid hormone, was augmented by [illegible] and possibly augmented acutely by intravenous mercaptoimidazole.

Interest in complications of I^{131} therapy has continued. A case of leukemia associated with I^{131} therapy of thyroid cancer has been studied and is being reported with details of dosage.

Future Studies - The kinetic studies of the effects of various hormones on protein metabolism will be extented. We also plan to perform detailed and prolonged studies of thyroid, plasma, urine, and fecal I^{131} to determine insofar as possible (a) the compartmental model best fitting overall thyroid and thyroid hormone metabolism, (b) the effects of various agents on the rate constants of the model.

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Lewallen, C. G., Rall, J. E. and Berman, M.: Studies of Iodo-albumin Metabolism. I. A Mathematical approach to the Kinetics J. Clin. Invest. 38: Jan. 1959.

Lewallen, C. G., Berman, M. and Rall, J. E.: Studies of Iodo-albumin Metabolism. II. The Effects of Thyroid Hormone. J. Clin. Invest. 38: Jan. 1959.

Serial No. 136-C
1. Clinical Investigations
2. Clinical Endocrinology Branch
3. Bethesda

NIH-NIH
Individual Project Report
Calendar Year 1958

Part A:

Project Title: Binding of Cortisol by Serum Protein.

Principal Investigator: Saul W. Rosen, M. D.

Other Investigator:

Cooperating Units:

Man Years (calendar year 1958): Patient Days (calendar year 1958)
Total: 1/2
Professional: 1/2
Other:

Project Description:

Studies are beginning on the amount of dialyzable hydrocortisone in normal serum. Using tritium labelled hydrocortisone and the technique of equilibrium dialysis, some preliminary data have been obtained. These show that hydrocortisone in submicrogram amounts, is neither adsorbed to glass nor to a cellophane dialyzing membrane. When the steroid is added to serum inside a dialysis bag and the system shaken until equilibrium has been reached, only some 0.3% is unbound at 4°C., whereas ten times more, or roughly 3%, is free at 37°. This temperature dependence is quite striking and does not occur in the well studied protein interactions with charged species. At the moment, reversibility of the binding is under scrutiny. Once the range of unbound hydrocortisone has been determined for fasting normal subjects, I hope to study the diurnal variation of free F and its level in several pertinent clinical situations, viz. pregnancy, adrenal hyper- and hypofunction, and rheumatoid arthritis.

Experiments are also beginning in the attempted isolation of the hydrocortisone binding protein(s) by gradient elution on a diethylaminoethyl cellulose anion exchange column.

Part B included Yes ☐ No ☒

Record No. 237-2
1. Clinical Investigations
2. Clinical Endocrinology Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A:

Project Title: Chemistry of Polypeptides and Proteins.

Principal Investigator: Harold Edelhoch, Ph.D.

Other Investigators: Roland Lippoldt

Cooperating Units:

Man Years (calendar year 1958): Patient Days (calendar year 1958).
Total: 2-1/3
Professional: 2
Other: 1/3

Project Description:

The transition between α-helical (Pauling-Corey α-helix) and random coil forms of synthetic α-polypeptides as well as between native and denatured proteins is currently under intensive investigation in many laboratories using optical rotatory measurements. The optical rotation of α-polypeptide structures will be composed of the optical rotation of the individual amino acids and in addition an independent contribution will be made by an ordered arrangement (i.e., α-helix) of the asymmetric atoms. It seemed of importance to current concepts of protein structure to evaluate the optical rotatory behavior of a polypeptide that cannot form an α-helix. A study of this kind has been completed with natural γ-poly-D-glutamate obtained from the spores of B. anthracis.

It was found that the optical rotatory behavior of γ-poly-D-glutamate did not resemble that of α-polypeptides with respect to any of the variables tested. Large changes in rotation and in the Drude dispersion constant were observed as the pH was varied or as the solvent system was modified. The marked sensitivity of this γ-linked polypeptide to environmental factors was attributed to the fact that the charged carboxyl group is α to the asymmetric carbon atom, whereas in α-polypeptides (and proteins) all ionizable charged groups are, at best, β to the asymmetric carbon atom. In addition to pH and solvents having marked effects, various cations also produced major changes in rotation.

Part B included Yes [X] No [ ]

This study has highlighted the fact that other means than a coil $\rightleftharpoons$ helix transition exist for producing appreciable changes in the optical rotation of polypeptides.

An investigation of the molecular structure of thyroglobulin has been begun. Thyroglobulin has been extracted in good yield from calf thyroid tissue by conventional methods.

A rather detailed analysis has been performed of the sedimentation and viscosity properties of thyroglobulin as a function of pH and ionic strength. The interaction with anionic detergents (e.g. sodium dodecyl sulfate) has been studied also by these methods. It has been observed that thyroglobulin dissociates into smaller fragments at higher pH values. These fragments no longer behave as globular proteins but more closely resemble linear polyelectrolytes in their properties. This dissociative reaction appears to be partially, if not completely, reversible. Sodium dodecyl sulfate is bound to thyroglobulin and promotes dissociation and unfolding of the native molecule. The reaction appears to be controlled by electrostatic factors since both alkalinity and detergent binding increase the net charge and enhance the extent of reaction. Light scattering studies support the dissociative process deduced from ultracentrifuge experiments.

Finally, some additional experiments have been conducted on the effects of guanidine, $Pb(NO_3)_2$ and ethyl alcohol on the temperature coefficients of pepsin inactivation. All three reagents lower the heat and entropy of activation. Guanidine and $Pb(NO_3)_2$ probably react with carboxyl hydrogen-bonded groups. Ethyl alcohol cleaves hydrophobic bonds and thereby reduces the thermal requirements for denaturation.

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Edelhoch, H. and Lippoldt, R. E.: The Optical Rotatory Properties of Bacterial γ-poly-D-Glutamate. Biochem. Biophys. Acta, In Press.

Edelhoch, H.: The Denaturation of Pepsin. III. The Effects of Various Protein Denaturants on the Kinetics of Pepsin Inactivation. J. Am. Chem. Soc. In Press.

1. Clinical Investigations
2. Clinical Endocrinology Branch
3. Bethesda

NIH-NIH

Individual Project Report
Calendar Year 1958

Part A:

Project Title: Model Reactions for the Biosynthesis of Thyroxine and Thyroid Proteins.

Principal Investigator: Hans J. Cahnmann, Ph.D.

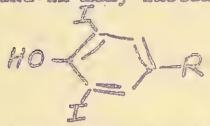
Other Investigators: Teruo Matsuura, Ph.D. and Annamarie Hofer, Ph.D.

Cooperating Units:

Man Years (calendar year 1958): Patient days (calendar year 1958):
Total: 2-1/3
Professional: 2
Other: 1/3

Project Description:

Influence of the structure of the side chain on the nonenzymic conversion of desamino analogs of diiodotyrosine to analogs of thyroxine: In order to determine the structural requirements for the conversion of analogs of diiodotyrosine to analogs of thyroxine, and in order to find a good model substance for the study of the mechanism of this reaction, the main products formed in the incubation of the following substances were determined qualitatively and in many instances also quantitatively:



a, R = $COOH$
b, R = CH_2COOH
c, R = $(CH_2)_2COOH$
d, R = $(CH_2)_3COOH$
e, R = $CH_2CH(CH_3)COOH$
f, R = $CH_2CH(C_6H_5)COOH$
g, R = $CH{=}CHCOOH$
h, R = $CH_2CH(OH)COOH$

Chloroacetyldiiodotyrosine was also incubated as a reference substance.

The investigation revealed that small changes in the structure of the side chain exert a considerable influence on the yield of the corresponding analog of thyroxine. The highest yield of thyroxine analog (11%) was obtained with diiodophloretic acid, e, (desaminodiiodotyrosine), the next highest (6%) with h, (hydroxy analog of diiodotyrosine). (Chloroacetyldiiodotyrosine gave, under the same conditions, chloroacetylthyroxine in a 1.5% yield). The facts that diiodophloretic acid forms an analog of thyroxine in particularly good yield and that in this case side reactions occur only to a small extent, should make it a good model substance for the study of the mechanism of this reaction.

Fate of the "lost side chain" in the synthesis of tetraiodothyropropionic acid by nonenzymic incubation of diiodophloretic acid: When two molecules of diiodotyrosine react with each other to form thyroxine, the alanine side chain of one of these two molecules is

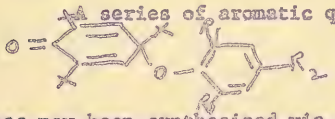
Part B included Yes [X] No [ ]

eliminated. The fate of this "lost side chain" has been a matter of controversy for many years. Since the study of the fate of this side chain offers considerable experimental difficulties (due to the large extent to which side reactions occur), the fate of the lost side chain in the case of the nonenzymic synthesis of tetraiodothyropropionic acid from diiodophloretic acid was investigated. It was found that the side chain is lost as two substances formed in about equal amounts, viz., hydracrylic acid (desamino serine), $HOCH_2CH_2COOH$ and dihydracrylic acid (the ether of hydracrylic acid), $HOOCCH_2CH_2OCH_2CH_2COOH$.

Mechanism of the formation of hydracrylic acid and dihydracrylic acid during the incubation of diiodophloretic acid- Since dihydracrylic acid is obviously formed from two side chains, the question arises whether two molecules of hydracrylic acid combined to form it or whether both, hydracrylic acid and dihydracrylic acid, originated from a common precursor. Of the possible precursors, acrylic acid (CH_2=CH COOH), propiolactone $\begin{pmatrix} CH_2\text{-}CO \\ CH_2\text{-}O \end{pmatrix}$, and the carbonium ion $^{+}CH_2CH_2COOH$, the first two were excluded because they did not form dihydracrylic acid under the conditions of the incubation experiment. For the same reason, hydracrylic acid was excluded as a precursor of dihydracrylic acid. It appears that the latter as well as hydracrylic acid are formed from a very reactive precursor, which probably is $^{+}CH_2CH_2COOH$, although this has not been proven directly.

According to Johnson and Tewkesbury and to Harington, the first step in the biosynthesis of thyroxine is a free radical reaction in which a quinol ether is formed. The formation of such a quinol ether intermediate could, however, never be demonstrated. Furthermore, aromatic quinol ethers have never been described in the chemical literature and, therefore, their conversion to diphenylethers (thyroxine) could not be investigated.

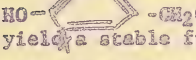
A series of aromatic quinol ethers

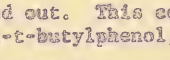


(✣ = t-butyl; R_1 = H, Br, I; R_2 = aliphatic side chain)

has now been synthesized via a free radical mechanism. Some of them have beenconverted by heat or acid catalysis to analogs of thyroxine. Thus the sequence of reactions carried out represents a complete model for the postulated mechanism of the biosynthesis of thyroxine. The analogs of thyroxine were tested for thyromimetic or antagonistic activity.

A free radical oxidation of di-t-butylphloretic acid,



was carried out. This compound does not yield a stable free radical (as tri-t-butylphenol,  does) but it was thought that perhaps the transient free radical would form tetra-t-butylthyropropionic acid via a quinol ether. No analog of thyroxine was detected in the reaction mixture. It seems that its formation is prevented by steric hindrance.

Investigations on Thyroid Proteins: Hydrolysis of thyroglobulin- The influence of concentration and temperature on the shape of the salting-out curve of rabbit thyroid proteins has been studied. C^{14}-labelled thyroglobulin which was 85% pure was prepared by the salting-out method. This was hydrolyzed and the hydrolysate was fractionated by chromatography according to Moore and Stein. It was hoped that a radioactive amino acid corresponding to the "lost side chain" could be found. This was not the case. Most of the radioactivity not due to tyrosine was found in the forerun containing the neutral and acidic constituents of the hydrolysate. If the side chain is split off as pyruvic acid or as hydroxypyruvic acid, the side chain radioactivity would be found in the forerun.

The Moore and Stein fractionation permitted the quantitative determination of the amino acid composition of the rabbit thyroglobulin. It differed greatly from the one published for other species. Glucosamine was also found in the hydrolysate.

Incorporation of iodide^{131} and of tyrosine-C^{14} into various thyroid protein fractions- In one series of experiments, I^{131} was incorporated into thyroid slices, alone as well as in the presence of not labelled iodide or of propylthiouracil. In another series, tyrosine-C^{14} was incorporated under the same conditions. Propylthiouracil exerted a strong inhibitory effect on the iodide incorporation into all fractions while iodide, at the concentration used, showed a lesser inhibitory effect and practically none on the incorporation into the particulate matter. Propylthiouracil as well as iodide had some enhancing effect on the incorporation into the thyroglobulin fraction which does not appear to be within the experimental error. Other fractions showed no major variations of the rate of incorporation of tyrosine in the presence or absence of propylthiouracil or iodide.

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

Matsuura, Teruo, Cahnmann, H. J.: Structural effects in the conversion of side-chain analogs of 3,5-diiodotyrosine to analogs of thyroxine. Biochim. Biophys. Acta 29:216, 1959.

Matsuura, Teruo, Cahnmann, H. J.: Model Reactions for the Biosynthesis of Thyroxine. I. Structural Influence of the Side Chain in Analogs of Diiodotyrosine on Their Conversion to Analogs of Thyroxine. J. Am. Che. Soc. 81: , 1959.

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

ANNUAL PROJECT REPORT

CALENDAR YEAR 1958

Summary Sheet

METABOLIC DISEASES BRANCH

Estimated Obligations for FY 1959

Total - $292,117
Direct - $178,000
Reimbursement - $114,117

Project Numbers 139-C thru 144-C Included

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Studies in Bone Metabolism

Principal Investigator: Dr. G. Donald Whedon

Other Investigators: Dr. Leo Lutwak

Cooperating Units: None

Man Years (calendar year 1958)
Total: 5-1/6
Professional: 1
Other: 4-1/6

Project Description:

Objectives: See report of 1957.

Methods Employed: See report of 1957.

Major Findings: 1. Metabolic balance studies: In five patients with osteoporosis, further metabolic balance studies have been conducted along the lines of a thesis which challenges traditional disregard for calcium intake as having a significant role either in the pathogenesis or in the repair of this prevalent disorder among older age groups. Three points are emerging more clearly in these studies: 1) patients with osteoporosis studied at the level of calcium intake they customarily consume are more apt to be in negative calcium balance than normal individuals taking the same level of intake; 2) patients with osteoporosis studied at increasing levels of calcium intake show increasingly positive calcium balances; at the highest level of calcium intake, the addition of gonadal steroids may not further increase the positivity of calcium balance; 3) variations in the data from one patient to another may reflect varying influences of a) level of nitrogen intake, b) level of phosphorus intake, c) calcium to phosphorus ratio in the intake, d) need for and/or administration of vitamin D; the importance of these accessory influences will need to be investigated in future studies.

In order to evaluate the effects of the adrenal steroid prednisolone without the complicating influences of disease, a normal male subject was given 25 mgm. a day of prednisolone for 40 days. Negative nitrogen balance developed as expected, but urinary calcium increased only slightly. The key analyses of stool calcium remain to be completed.

Part B included Yes [X] No [ ]

In a patient with myositis ossificans progressiva, long term balance studies were carried out to evaluate the effects of adrenal cortical steroids, hyperthyroid state, hypothyroid state, and hyperparathyroid state on the metabolism of calcium, phosphorus, nitrogen, and magnesium (data incomplete). In addition, intravenous disodium ethylenediaminetetraacetic acid was tried, unsuccessfully, in an attempt to modify therapeutically this progressive disease.

In Paget's disease, adrenocortical steroids have been found to induce a positive calcium balance, while relieving the subjective symptoms and reducing the serum alkaline phosphatase in this condition.

2. Measurements of bone formation rates using calcium-45 have been continued in patients with osteoporosis (10 studies in 4 patients), osteomalacia, acromegaly, myositis ossificans, familial hyperostosis, Paget's disease. The calculated data available to date confirm the findings reported in previous years. The study in recent months has proceeded to the evaluation of effects of hormones and dietary calcium on bone formation rate.

3. Methodology: a) A direct spectrophotometric method for the measurement of magnesium in serum, whole blood, urine and stool and diet extracts has been developed. Preliminary data suggest that adrenal corticosteroids have a primary action on the active renal excretion of magnesium that may be related to the apparent hypercalciuria sometimes seen in therapy with these agents.

b) A simplified calcium tolerance test has been evaluated. This involves the injection of 176 mgm. of calcium intravenously within one-half hour and measurement of the increased excretion of calcium in the urine in the 6 to 12 hours following the injection as compared with that for the same hours on the day prior to the test. Hospitalized subjects received constant diets the week prior to the test days; outpatients were instructed in a constant diet which was consumed for 3 days before and the days of the urine collections. Results have been obtained in 9 normal adults, as well as in patients with the following diagnoses: rheumatoid arthritis, osteoporosis, osteomalacia, osteitis deformans, osteogenesis imperfecta, hyperparathyroidism, myositis ossificans, acromegaly. The tests were performed at dietary intakes of 150 to 2400 mgm. of calcium per day and, in some of the subjects, under the influence of corticosteroids, estrogens, androgens, triiodothyronine, or vitamin D. In osteomalacia and osteogenesis imperfecta, the retention of calcium was high; in osteoporosis it was

within the normal range. Corticosteroids produced decreased retention, with increased retention 2 to 3 weeks after the steroid was discontinued. The most striking finding, in view of the increasing significance in our data of high calcium intakes in osteoporosis, was that in osteoporosis increasing the dietary intake of calcium increased the retention of the intravenously administered dose of calcium; retention was increased further by gonadal steroids.

Significance to NIAMD Research: See report of 1957. This year, the following may be added:

Minerals other than calcium and phosphorus are related to the process of mineralization through pathways as yet unknown. Magnesium, by virtue of its inverse physiological relationships to calcium, does not further evaluation.

Simplified tests for the ability of the organism to handle calcium, such as the modified calcium tolerance test, allow repeated evaluations of mineral metabolism in subjects likely to show changes, such as patients with rheumatoid arthritis receiving long-term therapy with potent corticosteroids.

Proposed Course of Project: It is planned to continue both metabolic balances and isotopic studies, which can be carried out simultaneously in the same subject, in an effort to determine the effects and mode of action in calcium metabolism of adrenal cortical and gonadal steroids and of the level of mineral intake of the subject as modified by changes in diet and by the addition of vitamin D. The role of magnesium, if any, will continue to be evaluated by the technique of balance studies in various bone disorders. The effects of long-term therapy with the above mentioned agents will be followed by the use of the calcium tolerance test, alone, and in conjunction with the two other techniques. From these studies are anticipated increased understanding of the complex processes of bone formation and resorption.

PHS-NIH
Individual Project Report
Calendar Year 1958

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1) Heaney, R.P., and Whedon, G.D.: Impairment of hepatic [illegible] clearance by the 17-substituted testosterones. J. Lab. Clin. Med. 52:169, August, 1958.

2) Heaney, R.P., and Whedon, G.D.: Radiocalcium studies of bone formation rate in human metabolic bone disease. J. Clin. Endocrin. & Metab. 18:1246, November, 1958.

3) Lutwak, L.: The estimation of radioactive calcium-45 by liquid scintillation counting. Anal. Chem. (in press).

4) Whedon, G.D.: Present concepts of the physiology of bone in the aging human: influence of hormonal and other factors in osteoporosis. Proceedings of the Fourth International Congress of Gerontology. (in press).

5) Whedon, G.D.: Osteoporosis: atrophy of disuse. Special book publication of research conference "Bone as a Tissue" held at the Lankenau Hospital, Philadelphia, Pennsylvania, October 30-31, 19[illegible]. (in press).

Serial No. [redacted]
1. Clinical Investigation [illegible]
2. Metabolic Diseases Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Metabolic Effects of Adrenal Cortical Steroids

Principal Investigator: Dr. G. Donald Whedon

Other Investigators: Dr. Leo Lutwak

Cooperating Units: None

Man Years (calendar year 1958).
Total: 1-5/6
Professional: 1/2
Other: 1-1/3

Project Description:

Objectives: See report of 1957.

Methods Employed: See report of 1957.

Major Findings: Two new antirheumatic compounds were evaluated for their metabolic effects, both in two patients with active rheumatoid arthritis, and one, in addition, in a normal subject. The results were as follows:

1. Delta-1,9-alpha fluoro, 16-alpha-methyl hydrocortisone [illegible] (also known as Decadron, dexamethasone) was given to one patient for 12 days at a dosage of 2 mgm. per day, 12 days at 6 mgm. daily, and 6 days at 10 mgm. daily. A second patient received this substance for 6 days each at 6 mgm. and 10 mgm. daily. A third (normal) subject received the drug at 6 mgm. daily for 10 days. A distinct sodium diuresis was seen in all at the dosages of 6 and 10 mgm. daily; little if any changes were noted in potassium balance. In one patient a definite increase in nitrogen excretion was noted, until, on the 10 mgm. dosage and on a daily intake of 11.5 gm. of nitrogen, slightly negative balance was seen. Increasingly negative phosphorus balance was seen in all. With increasing dosage of the drug, increasing excretion of calcium was seen; the negative calcium balance [illegible]

Part B included Yes [X] No [ ]

for 6 days after the drug was discontinued; the increased loss of calcium was accounted for almost entirely, by increased fecal excretion. This finding with respect to calcium is in marked contrast to the findings with previous steroids tested. This steroid has a marked antirheumatic activity and probably will have a broad clinical application.

2. 16-alpha-methyl hydrocortisone (MK-117) was administered to one patient for 12 days at 50 mgm. daily and to another for 6 days each at daily dosages of 50 and 100 mgm. Marked sodium diuresis was seen in both patients, but no changes in potassium balance. Increased nitrogen excretion was produced at both dosage levels, but negative balancewas seen only at the higher dose. Increasing excretion of phosphorus was seen at both dosages. At 50 mgm. daily, little change was seen in calcium excretion, but at 100 mgm., a negative balance occurred, mainly as a result of increased fecal excretion. Because of poor antirheumatic activity, further clinical trials of this drug are not contemplated at present.

Significance to NIAMD Research: See report of 1957. This study is cooperative with "Trial of New Antirheumatic Drugs," NIAMD.

Proposed Course of Project: This project will be continued intermittently along the present lines as facilities permit, with particular stress on electrolyte effects of the new steroids and attention to the long-term mineral effects, particularly of those compounds, such as prednisone, prednisolone, and dexamethasone, which appear destined for broad clinical use.

PHS-431
Individual Project Report
Calendar Year 1958

Part B. Honors, Awards, and Publications

Publications other than abstracts from this project:

1) See publications under project report of "Trial of New Anti[illegible] Drugs," Arthritis and Rheumatism Branch.

Serial No. NIAMD-141-C
1. Clinical Investigations
2. Metabolic Diseases Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Total Energy Metabolism: Studies in Health and Disease

Principal Investigator: G. Donald Whedon, M.D.

Other Investigators: Drs. E. R. Buskirk, R. H. Thompson, R. Moore & L. Lennox

Cooperating Units: None

Man Years (calendar year 1958)
Total: 5
Professional: 3
Other: 2

Project Description:

Objectives: See report of 1957.

Methods Employed: See report of 1957.

Major Findings: 1. Influence of Cold Environment on Specific Dynamic Action (SDE). The principle of metabolic replacement or equivalence of thermogenesis induced by food intake (SDE) and by cold has been assumed since the original work of Rubner on dogs. Since the reason for equivalence of these two modes of heat production has remained obscure, the problem was re-explored in human subjects. At various environmental temperatures oxygen consumption was measured continuously for 5 to 6 hours with subjects a) fasting throughout and b) fed a 1000 kcal. liquid meal (40% of kcal. from protein). Cold environment (50°F) altered the oxygen consumption curve after eating (SDE), producing an earlier peak and a shorter duration than in a comfortable environment. Cyclic variation of oxygen consumption with shivering was prominent in all experiments in the cold. Comparing fasting experiments in the two environments, cold caused a doubling of the metabolic rate; eating in the cold had no effect on the shivering pattern and further raised the metabolic rate. Thus, in contrast to the 19th century work on dogs, summation of SDE and cold thermogenesis occurred; thermogenesis after food supplemented cold thermogenesis and did not replace it.

2. Relationships between Exercise, Caloric Intake and Body Weight. Three normal young female volunteer subjects, who varied considerably in body composition, were subjected to ad lib liquid diets and to conditions of sedentary living for two weeks, one hour of treadmill walking per day for two weeks and two hours per day of walking for two weeks (total 6 weeks). This was done in order to determine a) the

Part B included Yes ☐ No ☒

efficacy of using a liquid diet in healthy young subjects engaged in hard work for extended periods of time, b) the time relationship between change in caloric intake after a change in energy expenditure, c) how well women tolerate rather arduous treadmill work day after day and their optimal work-rest relationship. It was found that body weight was well maintained on the liquid diet. Caloric intake did not change in a consistent fashion when energy expenditure was altered. The women tolerated the treadmill work rather well although a longer treadmill adjustment period (7 days) was necessary than for male groups of similar age. Work periods of 20 minutes duration with at least ten minute rests between proved to yield optimal work efforts as well as the most prompt recovery. The data gathered in this preliminary investigation will be valuable in formulating work programs for weight reduction regimens in female normals and patients with obesity.

3. Influences on Utilization of Food Components and Energy Production: Activity and Inactivity. Measurements have been made of the intravenous glucose utilization in normal volunteer subjects under controlled conditions of bed rest and physical training as part of inquiry into the importance of activity and inactivity in relation to metabolism in disease. Prior studies on this question have revelaed increased glucose utilization during acute exercise and decreased utilization of orally administered glucose in chronic diseases associated with inactivity. Intravenous glucose tolerance tests were performed at intervals in subjects on controlled dietary intakes during complete bed rest for 1 to 3 weeks and during vigorous physical exercise training for 2 to 6 weeks. Although a wide inter-individual variability of glucose tolerance was observed, in all subjects complete inactivity produced a marked decrease in glucose utilization within the first week with values in the diabetic range. Within one week after institution of physical reconditioning, utilization returned to base-line values followed in some individuals by increases in utilization coefficients to far above normal.

4. Instrumentation. Continued extensive testing of the Metabolic Chamber instrumentation was accomplished revealing several sources of difficulty and potential trouble. During the course of the SDE experiment it was found that small changes in metabolic rate (oxygen consumption) could be measured reliably if frequent calibrations of the analytical equipment were made. The necessity for frequent calibration imposed undesired restrictions on the use of the equipment. It was felt that the need for frequent calibration arose from unstable

amplification plus temperature and pressure variations. Appropriate changes in the instrumentation were initiated and are being incorporated into the "new" system. In addition, in the course of chamber experiments to date it became obvious that the chamber instrumentation produced a tremendous amount of data in a form relatively unmanageable by hand without laborious effort. It was felt that a data handling link with the NIH IBM 650 computer facility would be the solution. An extensive review of data handling systems amenable with the chamber instrumentation was conducted, the results of this review have been synthesized, and specifications for bids on a data handling system are being prepared.

Significance to NIAMD Research: Studies of human energy metabolism in the Metabolic Chamber are concerned with a variety of basic problems of physiology and metabolism and are thus directly related to the principal interests of NIAMD research.

The study on specific dynamic action and the influence thereon of cold environment represents investigation of a fundamental physiologic phenomenon of energy metabolism only the broad outlines of which are now delineated; this particular study inspected and by its results cast some doubt upon a basic assumption which has been held, without intervening inquiry since Rubner, one of the classical giants in this field. The increased knowledge obtained will, as always, require further investigative efforts.

The study of the effect of inactivity and reconditioning on glucose utilization is again an inquiry into basic phenomena related to energy metabolism. Of itself, as carried out to date, the study is preliminary to later more detailed measurements of energy expenditure in relation to intermediary metabolic shifts. Study of the metabolic influence of inactivity is important since enforced inactivity is an unfortunate accompaniment of many chronic diseases including rheumatoid arthritis, and, conversely, in diabetes exercise has been recommended as an adjunct to therapy. Many effects on carbohydrate metabolism attributed to diseases and to changes in therapy may in reality be phenomena related to or resultant from changes in activity.

The Metabolic Chamber project has also had the practical significance this past year of rendering collaborative service to several other groups either through its exercise (treadmill) facilities or through its metabolic laboratory, specifically to the group working on intermediary purine metabolism, to the Section on Nutrition (Study of riboflavin excretion as influenced by partial starvation and exercise) and to the NCI Endocrinology Branch (Effect of Chronic Exercise on Unesterified Fatty Acid Metabolism in Normal Individuals and in Patients with Cancer).

Proposed Course of Project: Studies will proceed along the lines suggested in the report; selected relatively circumscribed problems will continue to be undertaken until the instrumentation has reached full sensitivity and reliability accompanied by the ability to handle and interpret voluminous, continuously acquired data. Further information will be sought regarding SDE and cold induced thermogenesis, particularly at so-called critical temperatures for the human (77-80°F) below which metabolic rate begins to rise. Further insight into the influence of activity and physical conditioning on metabolic processes will be sought with measurements of oxygen consumption, carbon dioxide production, intermediates such as lactate and pyruvate and with studies utilizing radioactive glucose. Studies of obesity will be initiated with investigation of effects of exercise in conjunction with weight reduction on body composition, energy balance and work efficiency.

Serial No. NIAMD-142-C
1. Clinical Investigations
2. Metabolic Diseases Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Study of the Immunology of Blood Cell Deficiencies

Principal Investigator: N. Raphael Shulman, M.D.

Other Investigators: Dr. Thomas C. Bithell and Joel H. Feigon

Cooperating Units: None

Man Years (calendar year 1958)
Total: 3
Professional: 1-1/2
Other: 1-1/2

Project Description:

Objectives: See report of 1957.

Methods Employed: See report of 1957. In addition, clinical observations were made of the natural course and response to treatment of immuno-hematologic disorders.

Major Findings: Detailed analysis of the nature of antigen-antibody reaction involved in drug purpura and *in vivo* studies of the processes involved in the pathogenesis of drug purpura which were reported in 1957 provided a basis for studying other diseases which appear to be caused by similar immunologic mechanisms.

1. Idiopathic thrombocytopenic purpura (ITP): Application of principles derived from studies of drug purpura to studies of patients with ITP led to the following interpretation of the pathogenesis of ITP and its response to therapy: 1) Attachment of antibodies to platelets *in vivo* does not destroy them directly through lysis or agglutination but makes them more susceptible to physiological sequestration, 2) Thrombocytopenia and subsequent purpura reflects simply an imbalance between the rate of platelet removal and production; neither damage to megakaryocytes nor direct damage to capillary endothelium appear to be involved. 3) Splenectomy removes a very active site of platelet sequestration without altering the underlying disease. Participation

Part B included Yes [X] No [ ]

of other organs in sequestration may account for unsuccessful results of splenectomy. 4) Beneficial effects of steroid therapy can be attributed to increased platelet production alone; other possibilities such as primary effects on vascular permeability or antibody level do not appear to be important. The dose of steroids required to elevate platelet levels may be of significance in predicting response to splenectomy. 5) Serological tests currently used for demonstrating platelet agglutination in ITP may not have any particular diagnostic value or implications in therapy.

2. Hemolytic anemia associated with stibophen therapy. In 1955 J. W. Harris reported a case of hemolytic anemia associated with stibophen therapy which he found to be caused by an antibody which agglutinated or hemolyzed red cells only in the presence of the drug stibophen (a trivalent antimony compound linked to two substituted catechol rings). Because the RBC-drug-antibody system appeared to be so similar to the platelet-drug-antibody system which we had studied, it was of interest to determine the fundamental nature of RBC-drug-antibody complex formation and the possible role of complement in the reaction, just as we had done in the case of drug purpura. These studies have resulted in the following findings:

1) The complexes formed between RBC, stibophen, and the antibody of stibophen hemolytic anemia are similar in every way to the complexes formed between platelets, quinidine, and the antibody of quinidine purpura. 2) The experimental results with the stibophen antibody are in complete agreement with a reaction model described by us indicating that attachment of a stibophen antibody can be effected by 1, 2, or 3 stibophen molecules, depending on the concentration of stibophen; and that antibody molecules attached to RBC's with one or three stibophen molecules do not cause complement fixation, but antibodies attached with two stibophen molecules do cause complement fixation and may cause subsequent hemolysis of the red cells. When complement is fixed by antibody-stibophen-RBC complexes it is not fixed at the site of attachment of individual antibodies but is fixed between antibodies on the red cell surface, the red cells entering the reaction non-specifically by virtue of having sites on their surface appropriate for adsorption of stibophen-antibody complexes. 3) Treatment of red cells with trypsin alters their surface in such a way that they are capable of adsorbing at least twice as much antibody and fixing twice as much complement as untreated red cells and are thus made much more susceptible to hemolysis. 4) The complement-fixing reaction of RBC-stibophen-antibody complexes is markedly temperature dependent, taking place at 37° but not at 27° and is quite specific in that human complement or a human complement-like serum factor is required.

3. The pathogenesis and approaches to treatment of a newly recognized obscure form of purpura. Two years ago Drs. Gardner and Diamond described several patients who developed painful ecchymotic lesions when the patients' own red cells were extravasated or injected into their skin. The authors considered this disease to be the result of an unusual sensitivity developed to their own red cells and called it autoerythrocyte sensitization. Its pathogenesis and treatment has remained totally obscure and only 4 patients with this disorder have been reported in the literature.

During the past year we have studied a patient with a similar disease, although the manifestations in this case were much more severe than those reported thus far in that minute amounts of red cells (0.02 ml. of a 1/10 dilution of whole blood, i.e., one fiftieth the amount used by Gardner and Diamond) injected intradermally provoked very marked local pain and ecchymosis as well as severe systemic symptoms. Extensive study of this patient has led to the following conclusions: 1) The disease does not appear to be caused by a sensitivity to red cells per se, there being no evidence that the basic abnormality involves an antigen-antibody type of reaction, 2) The disease, rather, appears to result from a peculiar sensitivity to histamine released in the skin. Intradermal injections of an amount of exogenous histamine (1 microgram in 0.02 ml.), equal to the amount of histamine bound by the patient's skin in the injected site (50 mg. of skin), was found to duplicate precisely the spontaneous ecchymosis, pain and constitutional symptoms. In addition, the disease could be duplicated by substituting for an intradermal injection of red cells or histamine an intradermal injection of one of the numerous basic amino acids or the proteolytic enzyme, trypsin, all of which are known to release tissue histamine. The disorder was not the result of excessive tissue histamine or total histamine turnover, excessive serotonin, or diminished catecholamines, 3) Red cells extravasated intradermally appear to have concentrated on their surfaces substances which release tissue histamine. This was indicated by the finding that incubation of highly purified red cell stroma (inactive itself) with plasma (also inactive) led to formation of a complex between stroma and plasma factors which had the same potency as native red cells in provoking the ecchymotic and systemic reactions, 4) Therapy in an attempt to neutralize the effects of, or decrease the concentration of tissue amines, i.e., administration of large amounts of antihistamines, reserpine, and natural estrogens, have so far proved to be ineffective. Adrenalin in large amounts given subcutaneously relieved systemic symptoms but did not prevent local ecchymosis.

Significance to NIAMD Research: In the field of hematology, these immunologic studies are providing a progressively clearer understanding of the pathogenesis of blood cell deficiency diseases, including purpuras and anemias, indicating the important etiologic role of sensitivity and complex antigen-antibody reactions in these diseases.

Based on earlier work on quinidine purpura, these studies provide for the first time an explicit basis for interpreting the nature of idiopathic thrombocytopenic purpura and a rationale for further experimental approaches to more effective therapy for it.

The studies of hemolytic anemia associated with stibophen provide important evidence that the detailed model for immunoreactions involving complexes between antibody, cells, and haptene and the specific mechanism for complement fixation by such complexes reported by us are applicable to antigen-antibody reactions involving different types of cells, red cells as well as platelets. The studies therefore have numerous implications in various diseases of suspected sensitivity which cause cellular damage (e.g., idiopathic thrombocytopenic purpura, collagen diseases, nephritis, etc.). Additional findings, more specifically related to hematologic diseases, explain for the first time the effects of trypsinating red cells on their immunologic properties, a hitherto empirical observation of enhanced reactivity. The findings of marked temperature dependence and complement specificity help to clarify certain attributes of hemolytic antibody systems which previously had been inexplicable.

The newly recognized, obscure form of purpura defined in these studies differs from other known types of purpura and indeed may also differ from the disease autoerythrocyte sensitization described by Gardner and Diamond (for their patients were not sensitive to histamine). It is the first time that direct release of tissue histamine has been implicated as the primary factor in the pathogenesis of disease. These observations may have bearing on other forms of obscure purpura as well as on the etiology of various types of histamine reactions.

Proposed Course of Project: Studies of the pathogenesis and treatment of diseases caused by, or thought to be caused by, cell-haptene-antibody complexes will be continued along the lines described in the 1957 and 1958 reports; other blood diseases involving cellular deficiencies will be explored. Further studies will be made of the nature of the blood vessel abnormalities produced by histamine, of the nature of the substances adsorbed on red cell stroma which may release tissue histamine and of drugs which may be effective in the treatment of purpuric disorders similar to autoerythrocyte sensitization.

Serial No. NIAMD-142-C

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Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

1) Shulman, N.R.: Immunoreactions involving platelets. I. A steric and kinetic model for formation of a complex from a human antibody, quinidine as a haptene, and platelets: and for fixation of complement by the complex. Jour. Exper. Med. 107:665, 1958.

2) Shulman, N.R.: Immunoreactions involving platelets. III. Quantitative aspects of platelet agglutination, inhibition of clot retraction, and other reactions caused by the antibody of quinidine purpura. Jour. Exper. Med. 107:697, 1958.

3) Shulman, N.R.: Immunoreactions involving platelets. IV. Studies on the pathogenesis of thrombocytopenia in drug purpura using test doses of quinidine in sensitized individuals; their implications in idiopathic thrombocytopenic purpura. Jour. Exper. Med. 107:711, 1958.

Serial No. NIAMD-163-C
1. Clinical Investigations
2. Metabolic Diseases Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis.

Principal Investigator: N. Raphael Shulman, M.D.

Other Investigators: Drs. Thomas C. Bithell and Joel H. Feigon

Cooperating Units: Dr. John Z. Hearon, Head, Office of Mathematical Research. 11Z

Man Years (calendar year 1958)
Total: 3
Professional: 1-1/2
Other: 1-1/2

Project Description:

Objectives: To study the effect of proteolytic enzymes and proteolytic enzyme inhibitors on the conversion of prothrombin to thrombin *in vitro* and *in vivo*. To study the interactions and turnover rates of various coagulation factors in diseases of hemorrhage and thrombosis in order to define further the nature of the blood coagulation mechanism and the factors of significance in the pathogenesis of coagulation disorders.

Methods Employed: See report of 1957.

Major Findings: 1. Study of the effects of proteolytic enzyme inhibitors on the conversion of prothrombin to thrombin. In studying the nature of the anticoagulant action of purified proteolytic enzyme inhibitors, in collaboration with Dr. J. Z. Hearon, it was found that the presence of proteolytic inhibitors in a system containing

Part B included yes ☐ no ☒

prothrombin and biological factors which convert prothrombin to thrombin (thromboplastin, accelerators, calcium) permitted kinetic analysis of the formation of two intermediate products prior to formation of thrombin. One of the intermediates was found to react with proteolytic inhibitors including one which we had been able to isolate from human plasma. The clotting inhibitor binds the intermediate in amounts which depend on the rate of the prothrombin-to-thrombin reaction and thus appears to act as a "buffer" in coagulation. The marked rate dependence would tend to prevent the type of slow clotting which might lead to intravascular thrombosis but would not interfere with the rapid clotting necessary to stop hemorrhage from an injured blood vessel. Another finding that helps explain how clotting is controlled was that only a small fraction of the available prothrombin can actually form thrombin; the size of this fraction is also determined by the rate of the prothrombin-to-thrombin reaction.

2. Study of the nature of the coagulation abnormality in hemophiloid states and the effect of calcium-binding agents on various blood clotting factors. Following the chance observation that plasma collected in the calcium-binding anticoagulant, disodium ethylene diamine tetraacetic acid (Na_2EDTA), was ineffective in the clinical treatment of patients with classical hemophilia (AHG deficiency) a study of the manner in which Na_2EDTA inactivated AHG was begun. It was found that not only Na_2EDTA, but all anti-coagulants which strongly bind calcium (e.g., oxalate, high concentrations of citrate, ion exchange resins) inactivate AHG, the rate and degree of inactivation being proportional to the affinity of the anticoagulant for calcium. In contrast, the factor involved in hemophilia B (PTC deficiency) was not inactivated by calcium-binding agents, nor was prothrombin or Factor VII. Factor V was inactivated in a manner similar to that of AHG. Other investigators had reported inactivation of Factor V by EDTA, their interpretation being that removal of ionic zinc or some other trace metal from solution interfered with an enzymatic step in blood coagulation. The present study indicates that AHG as well as Factor V are complexes or molecules containing calcium as an integral part of their structure, and that removal of calcium from these factors leads to irreversible inactivation or denaturation. In the case of AHG, the stability constant of the protein-calcium complex was found to be between 10^{-8} and 10^{-9}, on the basis of titration against various metal complexes of EDTA.

In order to study the effects of calcium-binding agents on AHG, PTC and Factor VII, precise methods of assay, permitting accurate measurement of as little as 5 to 10% differences in the absolute concentration of these factors, had to be developed. The techniques devised, which involve simultaneous *in vitro* and *in vivo* assay, not only permitted this degree of accuracy, but also provided information concerning the effectiveness of hemostasis provided by varying amounts of these factors given to patients with specific blood-clotting deficiencies. Of special interest were the findings that a constant fraction of any dose of each of the factors was inactivated per unit time and that the half life of AHG and Factor VII were both of the order of 7-8 hours while that of PTC was somewhat longer.

Significance to NIAMD Research: 1. The types of *in vitro* and *in vivo* coagulation studies being done represent a fundamental approach to the understanding of the nature of diseases of hemorrhage and thrombosis. These diseases comprise a major segment of hematologic disorders, which have been a categorical interest of NIAMD. Further progress in diagnosis and treatment of a number of hemorrhagic diseases depends on understanding the nature of the metabolism of various coagulation factors. Such studies are appropriate to this institute and have direct bearing on the understanding of protein metabolism in general.

2. In the studies of the effects of proteolytic enzyme inhibitors on prothrombin to thrombin conversion, the great value was demonstrated of collaborative mathematical analysis. The conclusions reached resulted from a combination of both experimental and mathematical analysis in which mathematics has not only proven the validity of the working hypothesis drawn from laboratory investigation, but has provided additional conclusions which have been confirmed experimentally. The mathematical analysis has also suggested new leads for further research.

3. In the studies of calcium-binding agents in relation to coagulation factors, the demonstration that at least two of the most labile coagulation factors contain calcium as an integral component suggests that further studies directed at relating molecular structure to function may prove valuable in unraveling some of the complexities of blood coagulation. It now appears possible to produce specific deficiencies of blood coagulation factors *in vivo* in laboratory animals using Na_2EDTA as an inactivating agent. If production of specific clotting abnormalities is successful, it will facilitate experimental analysis of the pathogenesis and therapy of the naturally-occurring disorders. The turnover rates which have been established for the several clotting factors studied provide a useful practical guide in clinical therapy.

Proposed Course of Project: Studies will be continued of the biochemical and biophysical characteristics of the reactions involved in conversion of prothrombin to thrombin, and investigations will also be continued of calcium-binding agents on blood-clotting factors with emphasis on relating molecular structure to function. The production in laboratory animals of specific blood coagulation factor deficiencies may be possible using mineral-binding complexes

1. Clinical Investigations
2. Metabolic Diseases Branch
3. Bethesda

PHS-NIH
Individual Project Report
Calendar Year 1958

Part A.

Project Title: Study of the Normal and Abnormal Physiology of the Formed Elements of the Blood.

Principal Investigator: Dr. Frederick Stohlman, Jr.

Other Investigators: None

Cooperating Units: None

Man Years (calendar year 1958)
Total: 2/3
Professional: 1/3
Other: 1/3

Project Description:

Objectives: Study of factors contributing to the production and destruction of formed elements of the blood in normal and disease states.

Methods Employed: Aside from routine determination of formed elements in the peripheral blood, these consist of measurement of red cell survival with Cr^{51} and differential agglutination, red cell production with Fe^{59} uptake, and platelet function with a variety of standard coagulation tests.

In addition, the ability of the marrow of patients with refractory type anemias to respond normally to standard stimuli, phlebotomy, hypertransfusion and steroids is being studied.

Assays for erythropoietine from suitable patients before and after various forms of treatment are being conducted in conjunction with our basic research project.

Major Findings: 1. A relationship has been established in clinical anemias between bone marrow cellularity, degree of hypoxia and plasma levels of erythropoietine. These observations are in agreement with experimental observations which indicate that at a

Part B included Yes [X] No [ ]

given level of hypoxia the plasma erythropoietine level is inversely proportional to the cellularity of the marrow. These findings are compatible with the concept of marrow utilization.

2. High levels of erythropoietine have been found in most although not all instances of chronic refractory anemia.

3. Erythropoietine has been found in the urine and studies thus far suggest a renal threshold.

4. It has been established that erythropoietine is not of importance in post-hypophysectomy anemia.

5. Evidence has been obtained suggesting that there is a second mechanism regulating red cell production, unrelated to erythropoietine. The mechanism postulated involves an inhibitor of red cell production produced in senescent red cells; when these cells are removed, as in hemolysis or hemorrhage, the inhibitor would also be removed and red cell production allowed to proceed at a more rapid rate. In experimental studies with rats, anemia was produced by bleeding, then the rats were placed in an atmosphere of 50% oxygen for 24 hours. Although increased oxygen concentration reduced the hypoxia and thus the erythropoietine level in the plasma, at the same time there was an increase in red cell production, indicating that the erythropoietine level was not the sole regulator of cell production. Clinical study of a patient with hereditary spherocytosis then produced additional evidence to support the second mechanism. The patient was maintaining a normal hemoglobin concentration in the absence of hypoxia and in the face of an increased rate of destruction of red cells (2 - 3 x normal). When a partial (50%) exchange transfusion was performed using normal cells, thus reducing the rate of cell destruction, the rate of cell production was correspondingly reduced. In view of the fact that oxygen delivery remained unchanged this study demonstrates that there is a second mechanism for the control of erythropoiesis independent of the hypoxia-erythropoietine mechanism.

Significance to NIAMD Research: Anemia is a common complication of arthritis and certain metabolic diseases and may be refractory to treatment. A better understanding of the regulation of erythropoiesis is of basic interest and should eventually result in improved therapy. The importance of establishing the role of the pituitary in the regulation of red cell production is apparent. Since a pituitary erythropoietic hormone has been postulated based on observations in animals, it became important, therefore, from the metabolic standpoint to establish in man whether the pituitary plays a primary role or is involved secondarily.

Proposed Course of Project: Continued study of the relationship of erythropoietine to the control of red cell production. Study of the suggested second mechanism of regulation of erythropoiesis independent of the hypoxia-erythropoietine complex, best exemplified by compensated hemolytic syndromes in which increased erythropoiesis in the absence of hypoxia compensates for increased rate of destruction of peripheral red cells. Study of such cases should help to clarify this second mechanism.

Part B: Honors, Awards, and Publications

Publications other than abstracts from this project:

1) Stohlman, F., Jr.: Observations on the physiology of erythropoietine and its role in the regulation of red cell production. Annals of the N. Y. Academy of Science. (In press)

ANNUAL REPORT

JANUARY 1 to DECEMBER 31, 1960

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASE

CLINICAL INVESTIGATIONS

ARTHRITIS & RHEUMATISM BRANCH

FURTHER OBSERVATIONS ON THE RHEUMATOID FACTOR

The biological nature and significance of the rheumatoid factor remains obscure. Controversy exists as to whether or not this heavy molecular (19S) macroglobulin is an antibody to serum 7S gamma globulin. It is not known whether the rheumatoid factor appears as a result of connective tissue inflammation and necrosis or whether it may play a role in the etiology and pathogenesis of rheumatoid arthritis. To illuminate some of these obscurities attempts have been made at the experimental production of the "rheumatoid factor" in animals. Rats infected with streptobacillus moniliformis developed suppurative arthritis and osteomyelitis. This microbial infection was associated with the appearance of a positive bentonite flocculation test and elevated sheep cell agglutination titer. Neither of these serological tests were positive in any of uninfected rats. Further work is in progress to pursue this encouraging lead.

Observations made in laboratories in Sweden, France, Poland, England and the United States have led to the inference that either the presence of a circulating rheumatoid factor or its inhibitor (a gamma globulin of smaller molecular dimensions) may be genetically controlled. Grubb in Sweden discovered that the normal population could be divided into two groups: one, whose serum has the capacity to inhibit the agglutination reaction of rheumatoid serum and rabbit anti-sheep erythrocytes, and the other whose serum lacks this inhibitory activity. The first constitutes 90% and the second 10% of the population. The inhibiting property is genetically determined by a single autosomal gene. By utilizing a technique originated in our laboratories (bentonite flocculation test) for detecting this phenomenon, we have been able to confirm Grubb's results in normal subjects living in this area of the country. The presence of rheumatoid factor (in contrast to inhibitor) in our native normal population occurs in 2-3% the same is true for European populations. Studies carried out in our laboratories on sera of 10% Negro[illegible] reveal that 12% showed a positive bentonite flocculation test. Further studies are in progress to determine the genetic significance of this unexpected finding.

In last year's report a new method was described for [illegible] factor in serum of patients with rheumatoid arthritis. This method, developed in NIAMD laboratories, employed sorbitol coated Newcastle Disease Virus and sheep erythrocytes. The method was devised primarily to measure the degree of purification achieved during the various steps in a procedure directed towards isolation of the rheumatoid factor. During the past year further attempts have been made to isolate the rheumatoid factor employing three different procedures. In the first procedure, a complex was formed between serum rheumatoid factor and heated normal human gamma globulin. The complex was recovered and dissociated with an acid buffer. The resulting solution was placed on a sucrose gradient and subjected to ultracentrifugation. The high molecular weight rheumatoid factor was recovered from the bottom fraction. This procedure resulted in a preparation with several hundred-fold increase in specific activity (measured by the Newcastle Disease Virus inhibition procedure) compared to the starting serum.

A second approach employed Cohn method for fractionating serum. The fraction containing the rheumatoid factor was then placed on a carboxymethylcellulose (CMC) column. Subsequent elution with buffer solutions produced a fairly pure preparation of rheumatoid factor. However, contaminating serum proteins were still present.

A third approach involved a one-step method of serum fractionation (Sarorff). Material obtained by this procedure was further fractionated on a CMC column. Again considerable purification of the rheumatoid factor was achieved, but contaminating proteins were not entirely removed.

STUDIES ON A NEW, HIGHLY POTENT, SYNTHETIC CORTICOSTEROID, DEXAMETHASONE

Sarett and his coworkers at Merck Sharp & Dohme have introduced an alpha and beta methyl radical at the C 16 position of the 9 alpha fluoro-prednisolone molecule. This compound has been studied at NIAMD since the beginning of 1958. In man, it has been found to be the most potent anti-inflammatory steroid thus far produced, being 25-30 times more potent than hydrocortisone and 6 times more potent than prednisone on a mg. to mg. basis. Its capacity to suppress corticotropin secretion in man is relatively greater than its anti-inflammatory potency. A single dose of 1.0 mg. of dexamethasone was sufficient to reduce the plasma hydrocortisone level to zero, in normal subjects. (Thirty mg. of

prednisone was necessary to produce the same effect.) Yet this compound did not suppress aldosterone production, antagonize aldosterone effects on renal tubular function or impair aldosterone responsiveness to sodium loss or deprivation. Complete metabolic balance studies indicated that dexamethasone did not cause retention of sodium or loss of potassium. At higher than therapeutic dosage, namely 6.0 mg. daily, this steroid produced a negative calcium and phosphorus balance which resulted from a significant increase in both urinary and fecal excretion.

Clinical trials done on 22 patients with rheumatoid arthritis followed in most instances for longer than 6 months revealed some interesting results. The functional status of most patients was considerably improved following dexamethasone therapy. For example, 12 of the 22 patients had been moderately or totally incapacitated. On the new therapy none were totally and only two were moderately incapacitated. On the other hand, none of the patients had been able to do normal daily physical activities without discomfort and with the new steroid 11 patients advanced to this class. Of 13 who had been receiving prednisone therapy and were shifted to dexamethasone, 8 showed moderate or marked improvement, 4 mild improvement and one patient failed to improve. Of 17 patients followed for longer than 6 months on dexamethasone 10 showed progressive reduction in dosage requirement. In 5 of the 10 the decrease in dosage was 50% or greater. This phenomenon is unusual in corticosteroid therapy. The three major adverse effects of prolonged corticosteroid therapy in patients with rheumatoid arthritis are: appearance or activation of peptic ulcers, compressed fractures of vertebral bodies and mental disorders. In none of the patients in our series has a peptic ulcer appeared thus far. In none have we observed mental changes, psychosis or hypertension. In 2 patients (both males) compressed fractures of multiple vertebrae occurred. Both patients had severe rheumatoid arthritis, were utilizing relatively high doses of dexamethasone and had been previously receiving high doses of prednisone for several years. None of the 22 patients developed hyperglycemia or glycosuria.

OTHER BIOCHEMICAL AND PHYSIOLOGICAL STUDIES ON ADRENAL CORTICAL STEROIDS

In the 1957 annual statement it was reported that the addition of prednisolone to liver homogenate resulted in a marked stimulation of conversion of glucose-C^{14} to $C^{14}O_2$.

This activity in a cell-free tissue preparation had not been demonstrated previously. These studies on the *in vitro* effects of corticosteroids on carbohydrate metabolism were extended in 1950. Glucose oxidation - Using C_1 and uniformly labeled glucose [illegible] and the production of $C^{14}O_2$ from [illegible] was found to be stimulated by cortisone when incubated in the presence of various steroids in concentrations as low as 5×10^{-9} M. Addition of large amounts of hexokinase served only to enhance the effects, and similar stimulation was observed with glucose-6-phosphate as substrate. Furthermore, direct assays of hexokinase failed to reveal any steroid stimulation. Such experiments were [illegible] glucose was presumed to be oxidized almost exclusively via the TPN specific hexosemonophosphate shunt. Finally, working with ultracentrifugate of rat liver it was possible to show that stimulation of CO_2 production from glucose-6-phosphate resulted from steroid A ring reduction by the mechanism of TPN production. (Steroid reduction is TPNH specific.) Steps in proof included (1) abolishing the effect with tremendous excesses of TPN, (2) correlation of individual steroid reductases with the degree of stimulatory effect shown by the steroid, (3) stoichiometry of the overall reactions. Furthermore the hexose-monophosphate shunt was not stimulated in tissue lacking steroid reductases. Lactate and Pyruvate Oxidation - $C^{14}O_2$ production from C_2 labeled (and to a lesser extent labeled) pyruvate and lactate by rat liver homogenate was significantly enhanced by cortisone and testosterone in concentrations as low as 10^{-7} M. Addition of TPN was essential for such an effect and ATP in high concentration would abolish the effect. Interestingly, liver homogenates from fasted animals either showed no effect by steroid or showed inhibition. Preliminary screening with a large group of steroids did not show good correlation with steroid reductase activity and stimulation of lactate and pyruvate oxidation, or the potential action of the steroid as a transhydrogenase co-factor. These studies may help elucidate the mechanism of action of adrenal cortical steroids and the role they play in regulating the rate of certain enzymatic processes of widespread biological importance.

Urinary steroid excretion studies in man. [illegible] cortical Steroids. Because of the need for sensitive and specific methods of assay of steroids in biological [illegible] further work was carried out on extending and improving these physical isotopic methods of assay. The availability of isotopes of higher specific activity made possible improvements in the assay of urine and adrenal vein [illegible]

studying the aldosterone secretion rate from the adrenal vein pouch during the cross-circulation it was possible to clearly demonstrate the presence of a trophic hormone for aldosterone that was not corticotropin, viz. increased rate of secretion of aldosterone with no associated increased rate of secretion of cortisol or corticosterone.

Protein Steroid Binding. There is considerable evidence that the adrenocortical steroids are transported in the circulation bound to proteins. It therefore was of interest to determine the fraction of the steroid that was protein bound and the fraction that was circulating free since it may be that only the latter fraction possesses biologic activity. Studies were initiated using both ultrafiltration and dialysis equilibration techniques. The preliminary data have served to confirm the data as obtained by other investigators, namely, that in studies carried out at 4° C more than 99% of the steroid is protein bound, and at 37° C approximately 96 to 97% of the steroid is protein bound at physiologic concentrations of cortisol (5 to 25 μg.%). At greater than physiologic concentrations only 70 to 80% of the steroid is protein bound. Following estrogen therapy there is an increase in the concentration of the protein-bound steroid so that at plasma cortisol levels of up to 60 μg.% more than 95% of the cortisol remains protein bound. Perhaps this observation explains the lack of biologic effectiveness of the elevated levels of cortisol seen following estrogen therapy.

GOUT

Metabolism of Uric Acid Ribonucleoside in Mammalian Tissues. In man and other mammals uric acid is produced by the oxidation of xanthine in the presence of xanthine oxidase. The detection of an alternate enzyme-catalyzed reaction by which uric acid arises, was described in the 1957 report of this project. Since then the nature of the reaction has been defined more precisely, and many properties of the enzyme have been studied. Of potential interest in relation to the pathologic physiology of gout is the observation that the alkaloid colchicine, a specific remedy for the acute arthritis of this disease, is a competitive inhibitor of the enzyme.

The enzyme, which was named uric acid ribonucleoside phosphorylase, catalyzes the reaction:

(1) Uric acid ribonucleoside + orthophosphate ⇌ uric acid + ribose-1-phosphate.

[illegible] and the final preparation failed to release the [illegible] of adenine, hypoxanthine, guanine and xanthine. Although uric acid ribonucleoside phosphorylase and inosine phosphorylase were clearly shown to be separable, thymidine phosphorylase activity persisted in constant proportion to the uric acid ribonucleoside enzyme at each step of its purification. It has not yet been determined whether the two enzyme activities are distinct or identical.

The reversal of reaction (1) could not be demonstrated with certainty despite the use of a wide variety of reaction conditions. If the reaction is reversible, at the equilibrium point ribonucleoside cleavage is favored. With inosine and its analogous enzyme, synthesis is favored.

Uric acid ribonucleoside phosphorylase is widely distributed among the tissues of rabbits and is most abundant in bone marrow and intestinal mucosa, two tissues undergoing active cell division. HeLa cells, also actively dividing cells, contain readily detectable levels of the enzyme. It has been found in tissues from beef, dog, guinea pig, hamster and man.

The purified dog preparation was inhibited by p-chloromercuribenzoate in low concentration, and this inhibition could be prevented by the presence of cysteine. These observations suggest that enzyme activity is dependent on the sulfhydryl groups of the enzyme protein. Many other inhibitors of the enzyme were found, including colchicine, a sulfoxide derivative of phenylbutazone, and phenylbutazone, listed in decreasing order of inhibitory capacity. Benemid and sodium salicylate failed to inhibit. By use of Lineweaver-Burk plots it was found that colchicine is a competitive inhibitor of uric acid ribonucleoside phosphorylase, and that the enzyme has the same affinity for colchicine as it does for uric acid ribonucleoside. Under conditions permitting 70% inhibition of uric acid ribonucleoside cleavage by colchicine, inosine phosphorylase activity was inhibited only 4%.

The results of the studies in this project suggest that in mammals the oxidation of xanthine is not the sole pathway for the production of uric acid. Whether the alternate pathway described above plays an important role in purine metabolism cannot be decided until the metabolic origin of uric acid ribonucleoside is elucidated. This is an important future objective in this project. Although the occurrence of uric acid ribonucleoside in human erythrocytes was reported

in 1922, this finding could not be confirmed by [illegible] investigators. We attempted to detect this ribonucleoside in erythrocytes from normal subjects and one gouty patient and were unable to do so. It appears, therefore, that if the ribonucleoside occurs in human tissues, it probably occurs only in trace amounts, not unlike many other intermediate compounds in metabolic sequences. It may be possible to demonstrate that in animals colchicine poisoning will cause the ribonucleoside to appear in the urine or accumulate in the blood and if this could be shown, then one could study urine and blood from gouty patients receiving colchicine to the point of toxicity. This is a second proposed extension of the project.

Another avenue of investigation suggested by this work concerns the question of whether inhibition of uric acid ribonucleoside phosphorylase is in any way involved in the therapeutic action of colchicine in acute gouty arthritis. One approach to this problem is to study whether other *in vitro* inhibitors of the enzyme have a beneficial effect on acute gouty arthritis. Propylthiouracil is a more potent inhibitor of the dog enzyme than is colchicine. In a preliminary study relatively low doses of this compound were administered to a patient who was in the midst of an acute attack of gouty arthritis. No beneficial effect was noted but further study is in order before any conclusions can be drawn.

Intermediary Purine Metabolism in Gout. Further studies of the metabolic origin and disposition of uric acid in gouty patients have been done by administering isotopically labeled precursors of uric acid along with labeled uric acid to patients with gout and hyperuricemia and determining the pattern and extent of incorporation of the isotope into urinary uric acid. In previous studies of Dr. Wyngaarden, six gouty subjects and one subject with essential hyperuricemia were reported to show consistently greater incorporation of glycine-1-C^{14} into urinary uric acid than did three non-gouty subjects. These findings were interpreted as evidence that the primary metabolic defect in gout is excessive production of uric acid. We have been able to show that although a substantial number of gouty patients do show incorporation of excessive amounts of this isotope into urinary uric acid, there are others who show an incorporation indistinguishable from that of normal non-gouty subjects, so that such excessive incorporation cannot be a defect characteristic of all gouty patients.

Pharmacologic agents which might possibly be useful in the suppression of the excessive synthesis of uric acid in gouty subjects have been investigated. Of a number of such tested, one that has shown to be effective is 6-diazo-5-oxo-L-norleucine (DON), a glutamine antagonist which has been shown by *in vitro* studies of other workers to inhibit [illegible]. One patient with severe tophaceous gout and polycythemia vera showed evidence of decreased uric acid synthesis as evidenced by a lower incorporation of glycine-1-C^{14} into urinary uric acid while receiving DON over a 5-day period. A second patient with excessive uric acid production and urate renal lithiasis showed a fall in his serum urate level from 8.6 mg.% to 6.5 mg.% and a drop in his daily uric acid excretion from 1369 mg/day to 730 mg/day in a 5-day course of therapy with DON. Such preliminary results give promise that pharmacological agents may be effective in controlling the excessive uric acid production seen in some gouty patients.

Studies on a More Potent Uricosuric Agent. Xanthine [illegible] the evaluation of therapeutic agents for management of patients with gouty arthritis has been continued. The new uricosuric agent, sulfinpyrazone (U-28315) reported in [illegible] further clinical evaluation in gouty tophaceous gout. In addition, studies have been made of the uricosuric action of zoxazolamine (Flexin) which has been in use for several years as a muscle relaxant and has been found by NIH grantees to have a very potent uricosuric activity equal to that of sulfinpyrazone. Even more important, the uricosuric activity of these two drugs is synergistic. In that the two drugs together produce a greater uric acid excretion than either drug alone. Such findings greatly extend the range of therapeutic action available to the physician in managing problem cases of gouty arthritis which have not responded to conventional treatment with probenecid, or who cannot take probenecid because of objectionable side effects. Although salicylates in low doses tend to suppress somewhat the uricosuric activity of zoxazolamine, preliminary studies indicate that at high dosages of salicylates, the uricosuric activity is additive to that of zoxazolamine. This is in contrast to the antagonistic action between large doses of salicylates and either probenecid or sulfinpyrazone, where salicylates negate the uricosuric action of these drugs in most patients.

FURTHER STUDIES ON ALCAPTONURIA, HOMOGENTISIC ACID AND PHENYLALANINE

Nature of Defect in Alcaptonuria. Quantitative assay of the enzymes involved in tyrosine degradation was made in kidney homogenates (mainly cortex) of alcaptonuric and non-alcaptonuric patients. Alcaptonuric kidney was found to differ from the normal only in having no detectable homogentisic acid oxidase activity; this enzyme was easily detected in the non-alcaptonuric kidney samples. We can now extend our earlier observations on the nature of the defect in alcaptonuria to include the lack of homogentisic acid oxidase activity in the kidney as well as the liver.

Enzymatic Synthesis of Homogentisic Acid. Detailed studies of the enzyme system of liver-forming homogentisic acid from parahydroxyphenylpyruvic acid (the keto acid of tyrosine) are being continued. We have found that vitamin C not only prevents an inactivation of this enzyme by its own substrate but it also affects the renal reabsorption of p-hydroxyphenylpyruvic acid. The observed retention of p-hydroxyphenylpyruvic acid by scorbutic guinea pigs is therefore partly due to a failure to metabolize this acid and in part to its deficient reabsorption in the kidney. The possibility that vitamin C has a similar effect in the human on the reabsorption of other acid metabolites is to be investigated.

Method for the Estimation of Homogentisic Acid in Urine. As the result of these investigations, a specific colorimetric method to measure small amounts of homogentisic acid in urine was developed [illegible]. This method will be useful not only as a more specific diagnostic test to differentiate homogentisic acid from other reducing compounds in urine, but will also permit the analysis of blood samples during tolerance tests with homogentisic acid.

Method for Estimating Plasma Phenylalanine. A specific assay method has been developed in this laboratory to measure phenylalanine enzymatically in plasma which is superior to any other methods now available. This method will be of value in diagnosing phenylketonuria (where plasma phenylalanine is elevated) and in the determination of plasma phenylalanine levels in tolerance tests employed to detect the phenylketonuric [illegible].

GENETIC POLYMORPHISMS IN MAN

Many biochemical traits in humans and other animals are genetically determined. Some of these may be classified as genetic polymorphisms; that is, the existence in a population of two or more easily distinguished forms of a trait, the lesser of which could not be maintained by recurrent mutations alone. The normal hemoglobin-sickle cell hemoglobin system, most of the blood groups, and other systems to be described below, fall within this classification. From studies in lower animals and theoretical considerations, there is reason to believe that selection may operate to maintain these traits in the incidences found and in some cases the selective forces may be related to disease. It is of interest to determine if populations living under different environmental conditions and prone to different diseases have different incidences of these genes. In addition, studies of the distribution of these traits can sometimes provide information on genetic relations between separated population groups. A field trip to Northern Alaska was made in the summer of 1958.

Haptoglobins: These are a family of serum proteins which bind hemoglobin. They occur in three major patterns in humans and their presence or absence is genetically determined. Blood collections from Eskimos and Athabascan Indians were made during the Alaska field trip. Bloods from Micronesians were supplied through the courtesy of Dr. J. E. Hall. It was found that Eskimos and Indians living under similar environmental conditions have distinctly different distributions of the phenotypes. The Eskimos have a much lower incidence of the type 1-1 than do European populations (Eskimos - 7%, Europeans - 15%) and a very much lower incidence than that found in West Africans during our field trip of 1957 (West Africans - 50%). Micronesians from the Marshall Islands have an incidence of type 1-1 of 41%, and Alaskan Indians approximately the same as Europeans. These differences may be explained in terms of "racial" dissimilarity as well as possible differences in selections for this trait. Several haptoglobin types, not previously described in man, were discovered in the Eskimo, Indian, and Micronesian populations. These may represent rare genetic [illegible]

The sera of several animal species have been investigated to determine if they possess haptoglobins and if so, if they form a polymorphic system. Bloods from approximately 100 Pribilof fur seals, Arctic ground squirrels and marmots were collected during the Alaska field trip. All these were found to have haptoglobins and intraspecies differences were found

in one area and mammal. By analogy to the findings in humans it is likely that these haptoglobin types are also genetic[illegible] has been initiated. They appear to have more than one haptoglobin type [illegible] studies and a genetic determination of the types demonstrated here may provide suitable subjects for the study of selection in the haptoglobin system.

Uricase - Hyperuricosuria and Gout in Eskimos: [illegible] has been found to excrete [illegible] and others that are excessive [illegible] uric acid is in part under genetic control. Some persons with leukemia and other cancers are also high excretors, but the genetic role in these cases is not clear. Individuals who excrete large amounts of UAUR are rare in European populations. Approximately 150 urine samples from Alaskan Eskimos and Indians were studied with the collaboration of Dr. A. C. Allison and Dr. Stanley Garrier. It was found that more than half of the Indians and one-fourth of the Eskimos are high excretors compared to only 10% high excretors in Europeans. The reasons for these differences are not known.

Taste Test: The ability to taste phenylthiocarbamide (PTC) and related compounds in certain concentrations is genetically determined, although the threshold distinguishing tasters from non-tasters may vary in different populations. PTC and related substances are chemically similar to many goitrogenic materials and it has been suggested that there is an association between the taste-non-taste polymorphism and thyroid disease. By means of a selection test using 7 serial dilutions of PTC the incidence of non-tasters in Eskimos was found to be 3%. [illegible] properly controlled study has been done on this population. The gene frequency is distinctly different from that in North American Indians and is another indication of the genetic disparity between the Indian and Eskimo. [illegible] work was done in collaboration with Dr. A. C. Allison.

The Primaquine Sensitive Red Blood Cell Trait: [illegible] to primaquine and related compounds leads to a self-limiting hemolytic crisis in about 10% of American Negroes. It has been found that this is due to a deficiency in glucose-6-phosphate dehydrogenase and other enzymes, and that the deficiency is under genetic control. This trait is also found in Mediterranean Jews [illegible] and makes them susceptible to [illegible] but is very rare in other Europeans. Studies in other [illegible] groups have not yet been reported. In collaboration with Drs. H. Kellermyer and A. C. Allison the presence of this trait in Alaskan aborigines was investigated. Two of 50 Eskimos studied were found to have the deficiency

Blood Group Studies: In conjunction with the other genetic traits the incidence of blood groups in Alaskan populations has been studied (in collaboration with Drs. F. H. Allen and A. C. Allison). Thirty-seven separate antigens were used. The findings, in general, confirm those of previous investigators. The incidence of the Diego factor was studied in these groups for the first time. This blood group is absent in Europeans and Africans but common in South American Indians and in lesser incidence, in North American Indians. It is also present in Japanese and Chinese populations and in some Southeast Asian groups. We found a very low incidence of this trait in Athabascan Indians (less than 1%). This is the lowest incidence so far found in any American Indian population. If these findings are confirmed in subsequent studies in North and South American Indian populations, it would suggest that a North-South line exists for this trait. It is possible that selection may have operated to cause this distribution.

CLINICAL ENDOCRINOLOGY RESEARCH

The principal areas of research of the Clinical Endocrinology Branch have as in previous years been carbohydrate metabolism and diabetes, thyroid biochemistry and thyroid disease and certain aspects of protein structure.

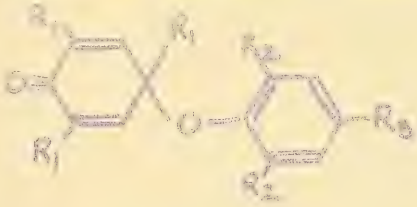
Carbohydrate Metabolism. Pursuing observations of Popper and Peach (vide supra) that progesterone and related steroids increase the rate of galactose C^{14} metabolism *in vitro*, the effects of progesterone on galactose induced cataracts was studied. In rats on a high galactose diet progesterone significantly inhibited cataract development. Further studies were done with galactose C^{14} in an individual with galactosemia. The inability of such individuals to convert C^{14} galactose to $C^{14}O_2$ was confirmed and a significant conversion to $C^{14}O_2$ was demonstrated after treatment with progesterone.

Studies of the effect of salicylates have been endemic to this branch for some time (see 1957 report) and recently effects of salicylates upon glucose metabolism were shown. In the isolated rat diaphragm salicylate increases the conversion of C^{14} glucose to CO_2 but has no effect on the net uptake of glucose by this muscle. An inhibition of muscle phosphorylase by salicylates has been demonstrated but is probably not the explanation for the increase in CO_2 production noted.

Work has continued on the role of insulin antagonists in the diabetic state. A modification of the rat hemidiaphragm test for insulin antagonist which is more sensitive has been developed. With this technique it has been found that most (of a small number) of individuals with acromegaly and diabetes mellitus had an insulin antagonist in their serum. Of interest was the finding that a certain number of previously untreated patients with diabetes had a serum insulin antagonist which disappeared after treatment. Other studies have shown that in several cases of massive clinical insulin resistance the antagonist demonstrated *in vitro* paralleled insulin requirement so treatment with adrenocortical steroids alleviated the resistance. Clarification of the types of insulin antagonists and characterization of their nature are being pursued.

Thyroid Biochemistry and Thyroid Di[illegible]

[illegible]tioned on the mechanism of thyroxine [illegible]. A mechanism previously suggested calls for the formation of an aromatic quinol ether as an intermediate in a free radical reaction [illegible]. This ether, however, never been described. It has been possible nevertheless, to prepare a series of such compounds of the general structure:



where R_1 = tertiary butyl

R_2 = H, Br, I

R_3 = aliphatic side chain

These were synthesized by a free radical mechanism and subsequently converted to diphenyl ethers (via thyronine analogues) by heat or acid catalysis. Several analogues of thyronine prepared in this manner and with the 3' 5' positions bearing tertiary butyl groups have been tested for biological activity and shown to be devoid of both thyromimetic or thyroxine inhibitor action. Further studies have been carried out to elucidate the fate of the aliphatic side chain which will be eliminated when one tyrosine molecule condenses to form thyronine. This was approached indirectly via tyrosine analogues since side reactions make a study with diiodotyrosine itself extremely difficult. Diiodotyrosine acid was condensed, tetraiodothyropropionic formed and the [illegible] chain found in 7 form via hydroxyllic acid /HOCH$_2$CH$_2$COOH/ and dihydroxyllic acid /HOCH(CH$_2$)$_2$ -O-(CH$_2$)$_2$COOH/. Indirect evidence suggested that the very reactive carbonium ion $^+$CH$_2$CH$_2$COOH is the intermediate which then forms both of the other substances.

Another and related interest has been the study of the isoprop[illegible] thyroglobulin. It has been found that in alkaline solution thyroglobulin dissociates into smaller molecules which no longer behave as globular proteins but resemble linear poly electrolytes. Anionic detergents such as dodecyl sulfate bind to thyroglobulin (Tg) and enhance its susceptibility to dissociation which is largely reversible. Electrostatic effects appear to govern this dissociation. In a related study the optical rotatory behavior of y poly D glutamate has been investigated. Since the alpha helix $\rightleftharpoons$ random coil transition has been intensively studied by optical rotation and rather sweeping conclusions drawn, it was of considerable interest to find that poly glutamate (which cannot form an alpha helix) showed marked changes in optical rotation (and the Cotton dispersion constant) with variations in pH and concentration of solvents and various cations. It must be concluded that factors other than the

ous of the intrinsic rotation of the asymmetric carbon atoms and the ordered structure of the entire molecule can affect optical rotation. This effect seems to be mediated through charge effects of the carboxyl group which is (in polyglutamate) alpha to the asymmetric carbon atom. In addition to Tg other iodoproteins of mammalian origin have been isolated and certain of their properties investigated. Two new types of iodoproteins have been described. One of these is unusually soluble at high ionic strength, appears to be a smaller molecule than Tg and has been named S_1. Kinetic studies suggest that in a certain strain of transplantable rat thyroid tumor it is the source of similar iodoprotein found in the serum. These rat tumors and another described below were generously provided by Dr. Seymour Wolman of the National Cancer Institute (see NCI report 906). A very similar protein has been found in the thyroids of certain individuals with goiter. Its significance in iodine metabolism and thyroid hormone synthesis is not yet clear. Another interesting iodoprotein has been found in large yield in one strain of rat thyroid tumor, in certain human goiters and to the extent of 5 to 15% (of I^{131} uptake) in normal human thyroid tissue. This protein is associated with the cell nucleus, and resists solubilization with 1N NaOH (which removes DNA) with 2N HCl (to remove histones). This protein (termed P_1) is rendered soluble by alkali (pH 13) and is found in the nucleus. After this treatment at least four separate proteins or polypeptides have been isolated from chromatography on derivatized cellulose. Two such components appear fairly homogeneous by ultracentrifugal analysis. Kinetic studies suggest that P_1 is neither a precursor nor product of Tg.

Work has continued on the mechanism of iodide concentration by the thyroid using, in part, a rat thyroid tumor and also normal human, rat and sheep thyroids. Several new inhibitors of the iodide trap, have been observed: $SeCN^-$ and certain digitalis glycosides such as digitoxin and strophanthidin. The latter two are of particular interest since it is known that they interfere with cation transport in muscle. For reasons, as yet unelucidated, these compounds are between 1000 and 10,000 times less active upon the iodide concentrating ability of the thyroid tumor than upon normal thyroid tissue.

Some further work on serum proteins which bind thyroxine has continued. A testosterone derivative (17 methyl 19 nortestosterone) has been shown to depress the level of serum thyroxine binding protein (TBP). The techniques for measurement of the level of TBP in serum have been revaluated using different buffers for electrophoresis. Bicarbonate buffer which reveals a pre-albumin component which binds thyroxine does not, however, alter the quantitative aspects previously developed for the measurement of TBP in serum.

Work on the adrenal cortical hormones has commenced. The binding of hydrocortisone to what appears to be a specific acidic protein in serum has been shown. The intensity of this interaction is such that 80% of serum hydrocortisone is bound. An unusual temperature dependence of binding has been noted.

The effect of hydrocortisone on protein metabolism using purified, autologous iodinated serum albumin has been studied. Surprisingly, no major effect upon either the degradation or transcapillary exchange rate of albumin as a result of treatment with hydrocortisone has been seen. The cooperation of members of the Rheumatics Panel has been particularly helpful in these kinetic studies. As before an extremely slowly exchangable compartment for serum albumin (in man) was seen. Studies done in rats to attempt to answer this observation on a smaller scale of the kind of this albumin might be performed were negative

METABOLIC DISEASES BRANCH

MINERAL METABOLISM STUDIES

Isotopic and metabolic balance studies of osteoporosis. Calcium metabolism studies reported last year revealed the dynamic nature of bone in that the tearing down and rebuilding of trabecular bone are demonstrated by isotopic studies in our laboratory to be a major turnover process, probably more important than ionic exchange. The unexpected finding that bone formation rate in osteoporosis appears to be normal, instead of reduced as current widely accepted theory would suppose, has forced the formation of a new hypothesis of bone metabolism. This new hypothesis accentuates the importance of minerals in the pathogenesis and correction of osteoporosis, a concept held by a minority (including ourselves) and based mainly on metabolic balance study findings. Studies this year, by isotopic techniques, have added to the evidence for this concept of the significant role of minerals in osteoporosis; important further information from isotopic studies of bone formation rate as influenced by calcium intake levels and by gonadal hormones, awaits completion of laboratory analyses. A highly important development in the field has been the recent confirmation of our finding of normal bone formation rate in osteoporosis by similar studies by Nordin in Scotland.

Development of short calcium tolerance test. A useful diagnostic test in bone disease has been the calcium tolerance test, introduced within the past decade and involving a four hour infusion of a calcium solution and collection of urines over 40 hours. Recently a Finnish, Lichtwitz, suggested a test involving a much shorter period of injection and collection for only 8 hours. Our laboratory has begun a comparative evaluation of the two forms of the calcium tolerance test and has applied the short version as a useful accessory method of evaluation of the state of the skeleton as affected by the various agents under study. The hormones investigated have generally brought about results as expected. An interesting and significant finding has been the observation that high calcium intake regimens appear to increase the avidity of the skeleton for mineral.

Metabolic evaluation of adrenal steroids. Metabolic evaluation of synthetic adrenal cortical steroids, carried out periodically as compounds of significant anti-rheumatic potency are found, has this year uncovered an apparently strikingly different steroid with respect to calcium metabolism. Dexamethasone has thus far appeared to be the most potent anti-rheumatic steroid; its relative freedom from serious electrolytic side-effects (no significant sodium retention or potassium loss) shown by this and other laboratories has permitted its release

steroids, except for a single instance in one patient metabolic studies have uniformly shown no calcium loss in overall balance; when urinary calcium increases have occurred they have been counterbalanced by decreases in fecal excretion. This puzzling difference between clinical and metabolic observation means that further studies must be conducted a) in normal subjects in order to rule out the complicating effects of disease, b) over long periods of time in order to see if the metabolic loss is merely delayed, and possibly c) at higher dose levels to determine whether the difference between dexamethasone and the other steroids can be resolved on the basis of comparable dosage. Investigation also needs to be continued of various possible ways of protecting the skeleton of patients who require steroid therapy for suppression of their rheumatoid arthritis.

their chapter on magnesium by stating that up to that time little clinical significance had been attached to changes in magnesium metabolism. In 1934, Hirschfelder described a syndrome of spontaneous tetany. However, it has been only in the past five or six years that any significant studies have been done on the role of magnesium in the body economy.

The distribution of magnesium is physiologically parallel to that of potassium. Biochemically, magnesium plays an essential role as a metallic activator of intracellular enzyme systems. Magnesium has been reported to be related to calcium and phosphate in the half of urinary excretion data. Parathormone is known to increase magnesium excretion. In some, but not all, cases of hyperparathyroidism and hypoparathyroidism, negative magnesium balances have been reported. Plasma magnesium concentrations have been variously reported to change in pathological conditions in a manner, parallel to, opposite to, or independent of, calcium and phosphate values. Recent studies indicate that measurements of intracellular magnesium would be of greater physiological significance. Preliminary reports from many centers indicate that magnesium may be related to diseases of bone, heart and nervous system.

The difficulty in accurately measuring magnesium in body fluids has been a serious limiting factor in studies thus far attempted. The older methods have been based on the precipitation of magnesium as the ammonium phosphate or hydroxy-quinolinate salt; these were accompanied by losses of magnesium in the separation of calcium. The most widely used technique at present is based on the formation of a compound with Titan yellow; this procedure is subject to interference by calcium and phosphate. Most recently techniques have been described based on titration with chelating agents, here again, calcium and phosphate interfere. Attempts at correcting these interferences make the estimation tedious and less accurate. The use of high temperature flame photometry has also been introduced; the lack of suitable instrumentation has hindered general use of this procedure.

We have devised a technique based on the formation of a colorless complex of magnesium with the brilliantly colored dye, 1-(2-hydroxy-1-naphthylazo)-2-naphthol-4-sulfonic acid. Under the conditions used, no interferences result from any substances present in serum, whole blood, urine, stool digests, or food. The technique is simple and rapid. It is hoped that this new procedure will be a valuable aid to investigators endeavoring to provide data concerning the role of magnesium in normal physiology and in various disease states.

HUMAN ENERGY METABOLISM STUDIES

Studies of human energy metabolism have thus far been limited to certain relatively circumscribed problems of basic energy metabolism. These have had merit in their own right as will be described below but have also served to define the capacity of Metabolic Chamber instrumentation and indicate where further improvements have yet to be undertaken. Predominant finding with respect to instrumentation has been realization of the extremely voluminous and intricate nature of the data turned out by the continuous stream gas analyzers and related physiological measuring devices. This has made it imperative to devise a data handling link through punch-paper-tape to the NIH IBM-650 computer. The latter facility will give valuable service in assembly, digestion and interpretation of Metabolic Chamber data information.

Influence of cold environment on Specific Dynamic Action. This study represents investigation of a fundamental physiological phenomenon of energy metabolism, only the broad outlines of which are now delineated. This particular study inspected and by its results cast doubt upon a basic assumption which has been held, without intervening inquiry, since Rubner one of the classical giants in this field. Whereas Rubner reported from study of two days that the heat produced following food would replace that eliminated by cold, our studies showed clearly in two normal subjects

that food taken in the cold had no effect on shivering and resulted in heat production over and above that already stimulated by cold. This study calls attention to the fact that a number of early and presumed fundamental observations in energy physiology may not apply to humans, may be based on insufficient experimental data or may contain inaccuracies which need correction by modern, more precise instruments.

Relationships between exercise, caloric intake and body weight. Studies have been conducted which are preliminary to investigation of normal subjects and obese patients for the effects of exercise in conjunction with weight reduction on body composition, energy balance and work efficiency. Liquid diets have been found feasible for studies involving hard work over extended periods of time. Young women were observed to tolerate treadmill work well although a longer adjustment period was required than for male subjects. Work periods of 20 minutes with at least 10 minutes rest proved to yield optimal work efforts.

Influence of activity and inactivity on glucose utilization. Measurements have been made of the intravenous glucose utilization in normal volunteer subjects under controlled conditions of bed rest and physical training as part of inquiry into the importance of activity and inactivity in relation to metabolism in disease. Prior studies on this question have revealed increased glucose utilization during acute exercise and decreased utilization of orally administered glucose in chronic diseases associated with inactivity. Intravenous glucose tolerance tests were performed at intervals in subjects on controlled dietary intakes during complete bed rest for 1 to 3 weeks and during vigorous physical exercise training for 2 to 6 weeks. Although a wide inter-individual variability of glucose tolerance was observed, in all subjects complete inactivity produced a marked decrease in glucose utilization within the first week with values in the diabetic range. Within one week after institution of physical reconditioning utilization returned to base-line values followed in some individuals by increases in utilization coefficients to far above normal.

Study of the metabolic influence of inactivity is important since enforced inactivity is an unfortunate accompaniment of many chronic diseases including rheumatoid arthritis, and, conversely, in diabetes exercise has been recommended as an adjunct to therapy. Many effects on carbohydrate metabolism attributed to diseases and to changes in therapy may in reality be phenomena related to or resultant from changes in activity.

Quinidine hemolytic anemia.

which agglutinated or hemolyzed red cells only in the presence of the drug, stibophen (or the ring component of its structure, sodium catechol disulfonate). Because the RBC-stibophen-antibody system responsible for hemolytic anemia appeared to be similar to the platelet-quinidine-antibody system responsible for drug purpura which we had studied, it was of interest to see whether the immune reactions of the two systems were comparable. The detailed model for the molecular interactions between platelets, drug haptens, antibody and complement reported by us last year was found to be precisely applicable to the reaction of the stibophen antibody with red cells. This model describes the steric and kinetic properties of cell-hapten-antibody complex formation and fixation of complement by the complex. The finding that different cell types react in the same specific manner with antibodies provoked by divers[illegible] provides a clearer understanding of the pathogenesis of various blood cell deficiency diseases and the numerous implications of suspected sensitivity involving cellular damage.

Studies of blood coagulation, prothrombin and its inhibition by proteolytic enzymes. In studying the mechanism of the anticoagulant action of partial proteolytic enzyme digests in collaboration with Dr. J. Z. Hearon, biomathematician, it was found that the presence of proteolytic inhibitors in a system containing prothrombin and physiological conversion factors (thromboplastin, accelerators, Ca^{++}) permitted kinetic analysis of the formation of at least two intermediate products prior to formation of thrombin. The intermediate which forms just prior to thrombin was found to complex irreversibly with proteolytic inhibitors. Including one [illegible] we had been able to isolate from human plasma. The amount of intermediate formed by proteolytic inhibitors is inversely proportional to the rate of prothrombin conversion. Such inhibitors may act physiologically as "buffers" in coagulation, momentarily preventing thrombin formation at slow rates of coagulation which tend to occur intravascularly but not interfering with thrombin formation when coagulation is accelerated extravascularly by tissue thromboplastin. Another interesting finding which has bearing on the physiological control of coagulation was the ability of prothrombin to be changed by biological conversion factors to inert protein as well as to thrombin, the fraction of prothrombin going up as thrombin being proportional to the rate of conversion. In these studies the great value was demonstrated of collaboratively mathematical analysis. The conclusions reached resulted from a combination of both experimental and mathematical analysis in which mathematics not only proved the validity of the working hypothesis drawn from [illegible]

for further research.

The mechanism suggested for fine regulation of red cell production. The existence of a second mechanism in the body (in addition to the erythropoietine system) helping to regulate red cell production and perhaps responsible for fine control has been suggested by recent studies. Past observations by other investigators have led to the conclusion that the sole regulator of red blood cell production is the state of oxygen saturation in the tissues. If the oxygen supply does not meet the demand, the production of red cells is accelerated and thus more oxygen becomes available. This acceleration is controlled by erythropoietine, a substance found in blood plasma which stimulates red cell production and which has been found to increase in quantity as hypoxia increases.

Clinical observations arguing against the hypoxia-alone theory are the body's response to minute blood losses, and the situation existing in cases of compensated hemolytic syndromes. In the first instance, if the hypoxia mechanism were the sole regulator of cell production, it would have to be extremely sensitive to hemoglobin concentration in the blood in order to respond to the minute change in this concentration resulting from the loss of only a few cc's of blood. The existence of such super-sensitivity is difficult to accept in view of the fact that daily fluctuations in plasma volume may produce alterations of as much as 6% in the hemoglobin concentration of the blood.

In compensated hemolytic syndromes there is a continual destruction of red cells, but no anemia occurs because of an increased capacity of the bone marrow to produce the cells. No hemoglobin deficiency is seen in such cases, and the increased cell production occurs in the absence of any hypoxia.

Recent NIAMD studies in rats have shown that red cell production in response to anemia caused by bleeding is unimpaired when the animals are placed in a high oxygen atmosphere. Clinical study of an autologous transfusion in a patient with hereditary spherocytosis has also shown that hypoxia need not be present for changes to occur in red cell production. It has been suggested that the second mechanism involves an inhibitor of red cell production which is produced in the senescent red cells and released on their death. Thus there would be a reduction in the amount of inhibitor released either when inhibitor is removed (as it would be by hemorrhage) or when less inhibitor is formed because of destruction of red cells by hemolysis before they reach senescence.

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

ANNUAL PROJECT REPORT

CALENDAR YEAR 1958

Summary Sheet

ADMINISTRATION

Estimated Obligations for FY 1959

Total - $95,000
Direct - $95,000
Reimbursement - 0

PHS-NIH
Individual Project Report
Calendar Year 1958

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Office of the Director
Bethesda

Part A.

Project Title: Administration

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Project Description:

Administration: Three major areas: (1) Basic Laboratory Research; (2) Clinical Investigations; and (3) Extramural Programs (Research Grants, Training Grants, Research Fellowships, and graduate medical training grants) comprise the major areas of activity of the National Institute of Arthritis and Metabolic Diseases. In addition, the Institute is responsible for providing business management services to the International Committee on Nutrition for National Defense, a world-wide survey activity. The Office of the Director is responsible for planning and directing the overall administration of the Institute, in conducting, fostering, and coordinating investigation of the cause, prevention, diagnosis, and treatment of gastroenterology, arthritis, rheumatism, and metabolic diseases; for maintaining effective operating relationships with other Institutes, with other units of the Public Health Service, with the Department of Health, Education and Welfare, other Governmental agencies, and public and private organizations carrying on related functions. The Office of the Director also participates in determining policies governing the National Institutes of Health.

During 1958, the Director, with the cooperation and advice of his staff, sponsored several cooperative conferences in collaboration with the American Diabetes Association, the American Rheumatism Association, and the Arthritis and Rheumatism Foundation. Chief among these were the Fifth Interim Scientific Session of the American Rheumatism Association which was held in Rochester, Minnesota, and the World Congress on Gastroenterology, which was held in Washington, D. C.

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

ANNUAL PROJECT REPORT

CALENDAR YEAR 1958

Summary Sheet

REVIEW AND APPROVAL OF GRANTS

Estimated Obligations for FY 1959

Total - $507,000
Direct - $268,000
Reimbursement - $239,000

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES
Annual Report - Extramural Programs

January 1 - December 31, 1958

Studies in arthritis and diabetes for which this Institute has had a long standing primary responsibility continue to be encouraged and advanced by the grants-in-aid programs for research and training for research. The more recently initiated programs of gastroenterology and physical biology are similarly benefiting by awards from the National Institute of Arthritis and Metabolic Diseases. It is rewarding to see the rapid yet logical development of the gastroenterology program recently undertaken to fill a long neglected void in medical research, while the evolving field of physical biology represents a highly desirable diffusion of the techniques and concepts of physics into biological problems. It is proving to be complementary to the aforementioned programs and holds the promise of serving as a sound base for the fundamental research pertinent to more clinically oriented investigations. In both old and new areas of interest, then, awards made during this year financed research in both clinical and fundamental aspects resulting in a well-balanced attack on the disease problems involved ranging from investigations of etiology and incidence to treatment and rehabilitation. Concomitantly the training programs supported will develop increased numbers of more thoroughly trained investigators and research motivated clinicians in the immediate future Budget increases in both training and research permitted conservative expansion of existing efforts while simultaneously enabling the initial support of some other worthwhile activities.

It is anticipated that these programs, especially those in gastroenterology and physical biology will be permitted to expand at a reasonable rate. It is also anticipated that the research programs for these new areas will facilitate, as is occurring in arthritis and diabetes research, closer cooperation between the clinical investigator and his counterpart in the basic disciplines

Research Grant Activities

A series of examples selected at random from the approximately 1300 research projects supported during the past year serve to highlight the nature and variety of this multi-faceted program.

In the field of arthritis, research on the rheumatoid factor has continued. A new analytical technique developed by Dr. Ralph Heimer and Olga Federico has revealed that the rheumatoid factor actually consists of a number of closely related protein substances instead of a single one. It appears not unlikely that the rheumatoid factor proteins might belong to a growing list of substances known as autoimmune bodies. Zoxazolamine, a drug widely used as muscle

relaxant, was [illegible] and to pro[illegible]
acid from the blood and is now undergoing clinical investigation. Its application in gout therapy resulted from an observation made during metabolic studies of the drug by Drs. J. J. Burns, T. F. Yu, Lawrence Berger, and Alexander Gutman. A new technique devised for experimental study of the healing process following bone fracture has been devised by Drs. J. H. Heller, H. Konig and L. Fox. This new in vivo method involves measurement of the bone uptake of radioactive strontium in rats with experimental bone lesions. It should have usefulness in a wide variety of experimental studies designed to evaluate various methods of therapy proposed to accelerate and improve the healing process in bone. Studies that promise to provide new insight into the processes of aging and [illegible] chronic degenerative diseases [illegible] under the direction of Dr. Paul M. Gallop. They reported the purification of an enzyme, collagenase, which breaks up the collagen molecule into fragments thus providing a tool for analyzing and determining its structure. The studies so far have revealed that one-third of the molecule is made up of glycine.

Research in diabetes has been characterized by intensive investigation of the hypoglycemic drugs. A clinical study of tolbutamide (Orinase) by Dr. Max Miller and co-workers has shown that the drug does not increase the uptake of blood glucose by peripheral tissue in the human body as insulin does. This is further confirmation that tolbutamide and insulin are not identical in their mode of action. A conference was held on the subject of chlorpropamide and diabetes mellitus. Dr. Stefan Fajans and colleagues reported that eleven diabetic patients poorly controlled by tolbutamide showed greater hypoglycemic response to chlorpropamide. The greater clinical effectiveness is due to its longer serum half life. This permits the attainment of a higher and more sustained level of serum sulfonylurea with much lower daily dosage. Dr. Piero P. Foa, in studying the action in dogs concluded that the hypoglycemic effect is due at least in part to a decreased glucose liver production and that there is no evidence for a pancreatrophic action. Almost all of the more successful oral diabetic drugs have been sulfonylureas. One exception is DB[illegible] a biguanide. In preliminary investigations, Dr. [illegible] Williams has found that this drug is preferable to tolbutamide or insulin in some patients, but more investigation will be required to determine its usefulness. A national conference sponsored jointly by the American Diabetes Association and the National Institute of Arthritis and Metabolic Diseases [illegible] the importance of diabetes training and the development of increased numbers of competent teachers and investigators in an effort to control the disease.

Developments in the field of gastroenterology have been extensive. Fundamental aspects of the ulcerative colitis problem were described and discussed in a special conference of [illegible] and clinical investigators and several possible fruitful areas of investigation were suggested. Another conference of interest in this field was the World Congress of [illegible] Washington, D. C. in May, 1958. A paper by Dr. Stewart Wolf and his colleagues described the isolation of biologically active

components of human gastric content by a new technique. For the first time a single fraction was obtained which contained two separate proteolytic enzymes--one active at pH 1.5 (pepsin) and the other at pH 3.5 (cathepsin). A device which is expected to contribute considerable new information on gastrointestinal physiology was described. Dr. Gaston Van Trappen presented a technique for obtaining motion pictures of continuous X-ray study of the intestinal tract over periods as long as 20 minutes. Dr. George B. Jerzy Glass discussed his investigations of the mechanisms governing the hepatic deposition, storage, and [illegible] of vitamin B_{12} in health and disease. An interesting development in the field of liver disease was the finding by Dr. James L. Gamble that the presence and kind of liver disease can be determined by measuring the relative concentrations of the two main bile acids in blood serum.

The disease, cystic fibrosis, has received increased attention and support from this Institute. Drs. Harry Schwachman and Marcel L. Kulczycki have recently completed a long-term study of 105 individuals with cystic fibrosis. Their study reveals that the effect of pulmonary involvement reflects the severity of the disease, inasmuch as the cause of death is eventually pulmonary insufficiency from a combination of diffuse obstructive phenomena and infection. The investigators believe their results indicate the effectiveness of the medical regimen employed and emphasize the value of pursuing a vigorous therapeutic program. This therapy consists of antibiotics given daily, pancreatin when necessary, and a high protein low fat diet. A new and more rapid method for measuring fat absorption in the body, suitable for evaluating fat digestive function in patients with cystic fibrosis of the pancreas, has been developed by Dr. Leroy W. Matthews. The new technique gives results which compare favorably with those obtained from older and more involved balance studies. When the test was applied to 11 children with cystic fibrosis, absorption of labeled fat averaged only 44% due to the diminished contribution of digestive enzymes from the pancreas. However, if pancreatin was added to the test meal, fat absorption increased in 9 of the patients to an average of 83%.

Of the 1,476 research grant applications recommended for approval, 1,293 have been paid or designated for payment in the amount of $17,428,670. These grants are distributed among 232 different institutions, in 47 states, the District of Columbia, Puerto Rico, Hawaii and six foreign countries. They support both clinical and fundamental research related to arthritis, various metabolic diseases, including diabetes and thyroid disease, liver disease, nutritional diseases, kidney diseases, gastroenterology and also research in physical biology.

As of December 1, 1958 there were 1,238 active research projects totalling $16,484,209, the average grant being $13 315.

	Requests		Approvals	
	No.	Amount	No.	Amount
March 1958 Council	347	$ 4,889,894	228	$ 2,772,109
June 1958 Council	1,040	15,237,802a/	918	12,864,461a/
Nov. 1958 Council	393	5,581,713	300	3,910,348
	1,780	$ 25,709,409	1,446	$ 19,546,918

a/ Reduced by withdrawals and refunds

Training Grant Activities

The widespread recognition of the need of training for both basic science and clinically oriented personnel for research in the traditional areas of responsibility of this Institute, continue to place increasing demands upon the limited resources available for such purposes. The new physical biology program has had the advice of a committee appointed for guidance in this area, and the program is now established on a firm basis. The gastroenterology program, also a new program has had a notable development. It should be emphasized that the potential demands for training programs remains essentially unsatisfied.

Active grants by category in 1958 were as follows: Arthritis - 30; Diabetes - 49; Gastroenterology - 20; Hematology - 13; General Metabolism and Endocrinology - 6; and Physical Biology - 6.

Requests		Approvals	
No.	Amount	No.	Amount
203	$ 4,164,898	193	$3,339,947

Of those recommended, 193 have been paid, or designated for payment, by the National Institute of Arthritis and Metabolic Diseases in the amount of $3,339,947. These training grants are to 71 different institutions, in 36 states, the District of Columbia, and Puerto Rico, and support 163 indirect trainees.

Direct Traineeships

These awards, by the Institute directly to physicians for support during advanced training, provide effective complementation to the training grants in providing a means for qualified individuals to obtain experience of a special nature. Although involving only a fraction of training funds, it contributes a highly desirable degree of flexibility to the training programs.

Requests		Approvals	
No.	Amount	No.	Amount
84	$ 384,734	78	$ 362,590

Of the direct traineeship applications recommended for approval 46 have been paid or designated for payment, by the National Institute of Arthritis and Metabolic Diseases in the amount of $232,[illegible]. These direct traineeships are to 28 different institutions, in 18 states, the District of Columbia, and England.

Research Fellowship Activities

The research fellowship program is an important component of the training program. By nature it is ideally suited to the training needs of the individual. It is complementary to the traineeship program in that it offers an opportunity for research training to the individual whose interests are in the area of basic sciences. It is a mechanism whereby the biologist, chemist or physicist is attracted to research endeavors essential to medical science.

Administration of the research fellowship program underwent reorganization to restore responsibility for the administration of the program to the Institute. This has resulted in much needed flexibility essential to adequate programming activity. It affords more nearly adequate information concerning various aspects of the program and has greatly expedited review approval and award procedures. Emphasis has been placed on the Special Research Fellowship Program as a means of most effectively utilizing the limited funds available to this program.

Type	Request		Awards	
	No.	Amount	No.	Amount
Predoctoral a/	81	$ 210,843	10	[illegible]
Postdoctoral a/	132	632,544	19	[illegible]
Special a/	29	187,775	11	[illegible]
Other b/			11	[illegible]
	242	$1,031,162	51	[illegible]

a/ These are estimates based upon the average award for each type of fellowship.

b/ Includes Post Sophomore and Foreign Research Fellows and Supply Grants.

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